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OTHO S. A. SPRAGUE MEMORIAL INSTITUTE

III

COLLECTED REPRINTS

VOLUME XI

CHICAGO
1923

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STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS

XXIV. THE ALIMENTARY ABSORPTION OF CALCIUM AND ITS DEPOSITION IN THE TISSUES IN EXPERIMENTAL TUBERCULOSIS

MARY E. MAVER AND H. GIDEON WELLS

From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, University of Chicago

Since calcified tubercles are usually either latent or sterile, healed tubercles, at least in man,[1] numerous therapeutic attempts have been made to stimulate calcification of tuberculous lesions with the idea that healing would thus be accomplished.[2] While we have no evidence that calcification favors rather than follows healing, it is certainly probable that a calcified tubercle is less likely to break down and liberate any remaining living tubercle bacilli than a tubercle that is not calcified. Whether administration of lime salts in any form or by any route will increase the rate of calcification is also problematical. Wersen (1) administered calcium lactate by mouth to 20 children with tuberculous peribronchial lymph nodes, and, with an equal number of untreated controls, studied them by fluoroscopy. He believed that a much larger number of the treated patients showed calcification of the nodes, as well as a greater improvement in health.

Of course, the resistance to tuberculosis of those who work in lime dust is traditional (2); but whether the calcium salts taken into the lung in these cases merely act mechanically to stimulate fibrosis, or are taken up and deposited in the tubercles, or whether they really have any beneficial influence, is not known.

The French, especially, have urged the value of calcium in tuberculosis, not so much with the idea of facilitating the calcification of tubercles, but on the hypothesis that in tuberculosis there exists a demineraliza-

[1] See Eugen Schmitz, Dissertation, Düsseldorf, 1909, and Wegelin, *Cor.-bl. f. Schweiz. Aerzte*, 1910, xl, 913.

[2] A review of the subject of the composition of calcified tuberculous lesions is given by Maver and Wells, AMER. REV. TUBERC., 1922, vi, 649.

tion, especially a loss of calcium. Robin, Ferrier, and others have written extensively on this subject, and Senator as early as 1877 stated that there was an excessive calcium excretion in the tuberculous. Sergent (3), who reviewed the early French work, was quite convinced of the value of calcium therapy. The calcium loss was supposed by some to be the result of intestinal lesions (Loeper and Béchamp (4)), while others have looked upon it as the result of a binding of the calcium by toxic products of the tuberculous infection (Croftan (5)). Most of the early clinical chemistry on which these ideas rested is inaccurate, and extensive deductions have been based upon such obviously valueless data as calcium determinations of the urine without consideration of either the calcium intake or fecal excretion of calcium. Ott (6), whose studies seem to have been more reliable than many others, found that with adequate nourishment there is no loss of calcium in phthisis; with undernourishment there is, of course, a loss of calcium as of everything else, but this is not an early feature of tuberculosis, or in any sense different from that occurring in cachexia or malnutrition from whatever cause.

A. Mayer (7) determined the balance of the inorganic elements in two severe cases of phthisis in twelve-year-old children and three adults. Although he found a marked increase in the amount of calcium excreted in the urine, this was more or less compensated by a decrease in the feces, so that in each case there was a small plus balance of calcium. There was also a lack of demonstrable evidence of loss of other inorganic elements, so that he failed to support the view that demineralization is an essential feature of tuberculosis. His general conclusions have been supported by Vannini (8) and by Labbé and Gallippe (9).

Voorhoeve (10) pointed out that the actual calcium balance in a tuberculous patient is of no significance, since decalcification is a relative term, depending on the amount of calcium supplied. He therefore studied carefully, in a few cases, the amount of calcium that was needed in the diet to maintain calcium balance, and found no very striking difference between his tuberculous and nontuberculous subjects. Certainly, if any tendency to decalification was present at all it was but little marked, but the number of cases was not sufficient to permit of drawing any conclusions. However, McCann (11) has stated that incomplete studies suggest that more calcium is required for maintenance of equilibrium in tuberculous than in normal subjects. The prevalent belief that consumptives suffer from dental caries because of abstraction of calcium could not be confirmed by Kiehnle (12).

Sarvonat and Rebattu (13), who have been advocates of calcium therapy in tuberculosis, give analyses of the tissues of four tuberculous and two normal guinea pigs, which, to quote from Kahn, they hold to prove that in the ash of the skeleton and soft tissues the content of phosphorus varies irregularly, tending to be high in the soft tissues. Their figures on the relative content of calcium of healthy and tuberculous tissues (guinea pig) are shown in the following table:

	NORMAL GUINEA PIGS	TUBERCULOUS GUINEA PIGS
	grams	*grams*
Calcium of bone	6.342	4.6
Calcium of soft parts	1.253	0.774
Relation of calcium to total (bone)	0.496	0.357
Relation of calcium to total (soft parts)	0.179	0.162

Max Kahn (14) says that he found tuberculous lung tissue twice or thrice as rich in calcium salts as normal lung, and that guinea pigs inoculated with tubercle bacilli showed a deposition of lime salts in the tuberculous foci; but no report of the details of this work have been published.

Loeper and Béchamp (15), however, found about the same amount of calcium in human lungs in tuberculosis as in other conditions, in the few cases examined by them.

Bracci (16), investigating the basis of the therapeutic use of calcium salts in tuberculosis, analyzed the lungs of rabbits to which calcium had been administered by various means, including intratracheal insufflation, and found the lung calcium always close to the normal. But rabbits that had received intratracheal insufflation of bismuth salts showed three times the normal quantity of calcium in their lungs, alike whether they were or were not given calcium. He stated his conclusions as follows:

1. The saturation of the organism of the rabbits with calcium salts in any form does not augment the amount of calcium in the lungs. 2. There exist substances in the lungs which have the property of fixing calcium in the lungs. 3. The substances which possess the property of fixing the calcium do not fix a greater quantity in the lungs of rabbits under the calcium cure. This fact agrees perfectly with the law that dominates the equilibrium of the organism. The proportion of the amount that is being assimilated is therefore invariable, and, as a matter of fact, part of the salts which are administered in excessive

quantities become eliminated without even being absorbed, part after absorption, and after their circulation through the body, are eliminated by the common ways of elimination, and part that are absorbed in the soluble form are rendered insoluble and are deposited in places where they can be gotten when needed for nutrition.

Steinitz and Weigert (17) analyzed the entire body of a year-old child, dead of tuberculosis, and found a normal total ash figure, and no abnormal distribution of the different inorganic elements of the ash, from which they inferred at least a lack of confirmation of the hypothesis that there is a demineralization in tuberculosis.

While the experiments of Tanaka and others show that injected lime salts may temporarily increase the calcium content of the blood and lead to metastatic deposition of calcium salts, yet it has not been demonstrated that feeding of these salts will modify the blood calcium in tuberculosis. Denis and Minot (18) did not find any increase of calcium in the blood of normal persons given calcium lactate by mouth, 6 gm. per day for six to ten days. Much calcium is normally excreted into the bowel, and hence it is quite probable that amounts appreciably raising the normal blood level will not be absorbed from the bowel. Nevertheless, as Givens (19) and others have found that feeding calcium salts causes an increased excretion of calcium in the urine, it seems probable that some absorption takes place in the alimentary canal, although the calcium may be so rapidly removed that it does not cause any appreciable protracted rise in the blood calcium. Thus Mason (20) found that a 5 gm. dose of $CaCl_2$ might cause a rise of plasma calcium by 2.5 mgm. per 100 cc. in three hours, but no effect was produced by calcium lactate.

Furthermore, a few observers have found that when considerable amounts of calcium are fed there may be some retention of calcium (Voorhoeve (21), Towles (22)) and increased calcium content of the tissues has been found in a few animals receiving calcium, studied by Kost (23). The accuracy of these older analytical studies may, however, well be questioned; thus, Voorhoeve used a method with an admitted error of 2.5 mgm. per 100 cc.

Howland and Marriott (24) found that when 0.5 to 1 gm. of calcium chloride was given to children with tetany, which is accompanied by a fall in the serum calcium from the normal, 10 to 11 mgm. per 100 cc. to from 4 to 7 mgm. (average 5.6 mgm.), there occurs a prompt disappearance of the tetany together with a rise of the blood calcium, some-

times to normal figures; but sometimes, despite continued calcium feeding, the serum calcium could not be sent above 7.5 to 9 mgm. Jacobowitz (25), however, was not able to increase the blood calcium by oral administration either in normal children or in those with tetany, even when the therapeutic results were excellent. Denis and Minot also observed that sometimes in animals with a low blood calcium the figure rose somewhat when calcium salts were fed, but normal figures could not be raised. Muggia (26), who could not find that there is any specific demineralization in tuberculosis, was also unable to raise the calcium content of the blood by administering inorganic calcium salts.

It is quite to be expected that figures lower than normal can be raised to normal, but that higher figures are not to be obtained, since we have it well shown by the phenomenon of metastatic calcification that the normal blood calcium represents about the maximum amount that the blood can carry, since greater amounts are promptly precipitated out in those tissues where the alkalinity is greatest or the CO_2 content the least.[3] The experiments of Heubner and Rona (27) show that even subcutaneous injection of nearly lethal doses of $CaCl_2$ (0.25 gm. per kg.) in cats produces a rise of only about 40 per cent in the blood calcium, reaching its maximum in from thirty to ninety minutes and falling to normal within five or six hours. Intravenous injections of maximal quantities may drive the blood calcium up to two or three times the normal, but the original figure is restored in about two hours, thus indicating the capacity of the body to restore the blood calcium to normal under even extreme conditions.

From the above review of the literature it is seen that *we have no experimental proof that the administration of calcium, either by mouth or otherwise, increases the amount of calcium deposited in tubercles or other necrotic areas*, unless we consider Wersen's clinical observation as experimental evidence. Nor, in view of the facts disclosed in metastatic calcification, should we expect any appreciable modification in the amount of calcium deposited in tuberculous tissues by changes in the calcium intake, except in those whose blood calcium is less than normal in amount. We know of no reliable figures by modern methods which show any deficiency in calcium in the blood in pulmonary tuberculosis. Halverson (28) and his colleagues have made careful analyses of the

[3] For a full review of the subject of metastatic calcification see Wells, *Arch. Int. Med.*, 1915, xv, 574.

blood in patients with pulmonary tuberculosis, and draw the following conclusions:

Determination of the calcium content of the blood of patients with advancing and convalescing tuberculosis revealed that in the incipient cases in which the patients, who were on a high milk diet, showed marked improvement, the values for calcium in the serum were normal and fairly constant. In no case was the calcium value increased above normal figures by this high calcium diet. In advanced cases the variations were greater (some rather high and some rather low values being obtained), and improving patients showed on the average slightly higher values than the unimproved. No marked deviations from the normal, however, were observed in the calcium content of the serum of patients in the various stages of pulmonary tuberculosis.

The failure of the body to deposit lime around the tuberculous areas is to be ascribed, not to a deficiency in blood calcium but rather to an inability of the cells of the tuberculous area properly to utilize available calcium. The total blood of a 60 kg. man contains about 0.3 gm. of calcium. The bones contain approximately 4,000 times as much. With this great reserve supply of calcium, a portion of which is available, as is indicated by the great constancy of blood calcium under varying diets and conditions, a physiological basis for the administration of calcium salts in tuberculosis has yet to be demonstrated.

While this last argument, as to the futility of giving calcium by mouth while the bones contain great quantities of calcium, is striking, it is not wholly convincing in the face of the existence of such conditions as tetany and some forms of nephritis, with a reduced blood calcium which can be restored to normal by giving calcium salts by mouth, with a coincident amelioration of the symptoms of calcium deficiency. Apparently, the bone salts are not always available for general metabolism.

In view of all this evidence, therefore, it seems *a priori* altogether doubtful that calcification of tuberculous lesions can be appreciably altered by oral administration of calcium salts. Whether a tubercle undergoes calcification or not would seem to depend chiefly, if not solely, upon how long it remains as a quiescent mass of colloidal organic material, presenting such physical properties as favor the deposition of calcium salts (29); that is, a human tubercle is probably at least latent before it begins to calcify. Furthermore, tuberculous lesions in cattle show partial calcification at all stages, yet seem to progress unimpeded by this process, and certainly bone tissue itself seems to be anything but resistant to tuberculosis.

It is, of course, perfectly possible that calcium is of value in the growth of connective tissue as silica is said to be, but we lack any proof of a shortage of calcium in tuberculous tissues, or of a greater capacity of connective tissues to grow in calcium-treated animals. Furthermore, the usual large daily intake of milk in the diet of the tuberculous patient guarantees an abundance of calcium.

Numerous clinical reports of favorable results from the use of calcium have been published, and are entitled to consideration, at least on the basis of their number. However, if there has ever been published an adequately controlled clinical study of the value of calcium therapy in tuberculosis we have not succeeded in finding it.

Although occasional writers on the clinical use of calcium refer vaguely to experiments which they have performed, specific reports are few. Van Gieson and Lynah (30) (31) have published enthusiastic approval of Russell's method of treatment (32), which is based on the doubtful theory that administration of hydrochloric acid to patients increases the amount of gastric rennin, which causes calcium to combine in an assimilable form with casein and have supported it only with observations on three dogs. Two of these dogs on a calcium-poor diet showed more tuberculous lesions than one on a normal diet.

Emmerich and Loew (33) call attention to Hamburger's demonstration that calcium salts make the phagocytes more active, and report experiments in which the resistance of guinea pigs to anthrax seemed to be increased by calcium feeding. Mice were also made more resistant against hog cholera by feeding calcium. Their experiments on tuberculosis consisted in inoculating 22 guinea pigs with tubercle bacilli of unspecified origin or virulence. Twelve of these had been fed 0.1 gm. per kilo of calcium chloride, daily, for from five to eleven months. The inoculation took place on the 18th of May and by the 27th of October two of the control and four of the calcium animals were alive, but all died before the 19th of November. The calcium guinea pigs in general held their weight better and showed somewhat less tendency to the presence of pulmonary lesions. As will be seen, the difference obtained was only one of degree and this was not constant. These authors speak also of the clinical use of calcium, mentioning a favorable influence on digestion exerted by calcium chloride, but the statements as to clinical results are very vague.

Massini (34) injected 10 rabbits with bovine tubercle bacilli and gave 1 cc. of 1 per cent $CaCl_2$ solution intravenously twice a week to half of

them. As these treated animals lived an average of six days longer than the other five he looks upon this as a "small success," although the range in the treated animals was from 75 to 120 days, and in the untreated from 68 to 116 days. It is difficult to interpret these figures as even a "small success." Michelazzi (35) infected 30 rabbits and guinea pigs, and found calcification of the lesions in rabbits treated with calcium, and in the guinea pigs a cirrhosis of the liver in the treated animals marked an increased resistance. As the calcified lesions in the rabbits were sterile he believed that he had obtained a curative effect. This work has not, as far as we can find, been repeated and confirmed.

Laitinen (36) reports two series of experiments (each on 20 to 30 guinea pigs), in which the animals fed calcium phosphate lived twice as long as the controls, but calcium hydroxide had no influence. Obviously, the duration of life in so small a number of animals as this has little significance in view of the great variations seen in any set of infected guinea pigs.

On the other hand, Leo (37) and Finsterwalder (38) observed no influence of calcium treatment on tuberculous guinea pigs. Kindborg found that calcium salts favor growth of tubercle bacilli in culture media. This was corroborated by Takenaka (39), who found that the calcium ion itself has *in vitro* no inhibiting action on the tubercle bacilli but rather favors growth, although some calcium salts are inhibitory. Of these he recommends trial of calcium salicylate, which causes an increase of both red and white cells when injected intravenously into rabbits. Intravenous injections of calcium chloride into four rabbits produced in three a diuresis with nitrogen retention, which he thinks may be a favorable factor in tuberculosis therapy.

Another way in which calcium may possibly be of influence is through its action on the leucocytes. Hamburger found that calcium ions have a strikingly positive chemotactic effect on leucocytes, which has been corroborated by Miss Wolf (40), but just how an increase in the calcium content of either the blood or the tissues as a whole could affect the action of leucocytes in a tuberculous lesion is not clear. Neither can we imagine an influence from calcium on the basis of the transitory rise in the proportion of polymorphonuclear leucocytes which follows injection of calcium salts (Rösler (41)). A general favorable effect of calcium on tissue metabolism has also been suggested.[4] However, the statistical

[4] See Starkenstein, *Therap. Halbmonatschr.*, 1921, xxxv, 553.

study of the influence of hardness of drinking water by Opitz (42) did not disclose any more favorable tuberculosis rates in populations drinking water containing much lime.

It must be freely admitted that when the experience of large numbers of clinicians, familiar with the course of tuberculosis in man, endorses the use of calcium as giving definite clinical benefit, we must listen with respect to this endorsement, even if we cannot explain such results or reproduce them in experimental animals. The resistance of workers in lime dust to tuberculosis is a world-wide belief,[5] and hence in all probability not groundless. On the other hand, the fact that most standard works on tuberculosis say little or nothing concerning calcium as an agent of value in the treatment of this disease would indicate that the enthusiastic opinions of the few have not been corroborated by the experience of the many.[6] Neither are negative clinical results lacking.[7]

A review of the literature by Max Kahn (43) concludes with this statement, "In looking over the mass of literature on this subject, one is left in doubt whether the use of lime in the treatment of tuberculosis is to be recommended."

Nevertheless, in view of the supposed clinical benefits, and especially the results reported by Wersen on the calcification of tuberculous lymph nodes under calcium therapy, it has seemed desirable to study experimentally the influence of calcium feeding upon the deposition of calcium in tuberculous and nontuberculous tissues under conditions that can be controlled.

THE CALCIUM CONTENT OF THE TISSUES OF NORMAL ANIMALS

The first analyses, for control, were made of the tissues of normal guinea pigs on the ordinary laboratory diet (oats, hay and carrots) and of normal guinea pigs given every day (except Sundays) each 0.188 gm. calcium lactate (containing 0.0338 gm. Ca) by mouth in gelatin capsules.

[5] See review by Tweddell, *Med. Rec.*, 1922, ci, 141.

[6] The use of calcium to check diarrhoea, as recommended by Maendl (*Ztschr. f. Tuberk.*, 1917, xxviii, 334; also *Med. Klin.*, 1920, xvi, 228), Ringer and Minor, AMER. REV. TUBERC., 1922, v, 876, and others, is not based on any action on the tubercle bacillus itself, or apparently on the tuberculous lesions, but depends on the effect of calcium on exudative processes, and is analogous to the use of calcium to increase coagulation in hemoptysis (Maendl, *Ztschr. f. Tuberk.*, 1921, xxxv, 184). This form of calcium therapy is not related to the use of calcium for a definite effect on the tuberculosis and is outside the theme of our discussion.

[7] See, for example, Petheö, who observed no benefit in 240 cases of juvenile tuberculosis. (Reference in *Zentralbl. f. Tuberk.*, 1922, xvii, 317.)

This dose is equivalent to one of 25 to 30 gm. calcium lactate daily to an adult human being.

Methods: Immediately after autopsy the tissues removed for analysis were dried of excess blood by wiping, and put into 95 per cent alcohol until analyzed, except for portions kept for microscopic study. For analysis they were removed from alcohol, cut into fine pieces, and dried to a constant weight at 110°C. It had been intended to analyze separately the tuberculous lesions and the nontuberculous portions of the same organs, but this was found to be possible in only a few cases. Since the amount of fat varies so much in the liver, this organ was extracted with ether in a Soxhlet extractor for six hours to remove this source of error. The bones analyzed were in each case a tibia and a fibula, which were washed in cold water and air-dried.

The tissues were completely ashed, and the ash dissolved in 0.5 N hydrochloric acid. The solution was filtered from possible minute carbon particles through a small ash-free filter paper (Whatman No. 30) into a 50 cc. centrifuge tube. Concentrated NH_4OH was added, drop by drop until the reaction was very slightly alkaline to phenolsulphonephthalein. The method described by Kramer and Tisdall (44) for the determination of calcium and magnesium in small amounts of serum or plasma, was applied with slight modifications to this solution. One drop of 0.1 N H_2SO_4, 2 cc. of saturated ammonium oxalate and 0.5 cc. saturated solution of sodium acetate were added, and the mixture was allowed to stand at least one hour. The volume of the mixture was made up to 8 cc., and the pH was about 5.4, an acidity which prevents the precipitation of calcium phosphate. The mixture was centrifugated for twenty minutes and washed with 2 per cent ammonium hydroxide. The washing and centrifugating were repeated twice. The precipitate was dissolved in 2 cc. 0.1 N sulphuric acid, heated on a boiling water bath, and titrated with 0.01 N $KMnO_4$, using a micro-burrette. (The large amounts of calcium in the bones permitted gross quantitative determinations.)

The results of these analyses are given in tables 1 and 2.

It will be seen that, while in the normal animals there is not a little variation in the calcium content of the same tissues in different animals and in the different tissues in the same animal, yet averaged together the amounts are not far from the same, ranging from 38 mgm. Ca per 100 gm. of liver to 52.8 mgm. Ca in 100 gm. of lymph nodes. The fact that lymph nodes generally contain more calcium than the other tissues probably depends on the inhalation of calcium dust, which will be particularly abundant in the cages of animals fed calcium, and its deposition

in the peribronchial and cervical lymph nodes.[8] If we consider that the tissues normally contain about 75 per cent of their weight as water, it will be seen that they contain about 10 to 13 mgm. of calcium per 100 gm. moist weight, or quite the same as the blood itself. Therefore, it

TABLE 1

Calcium determinations of organs of normal guinea pigs

NUMBER	WEIGHT OF ANIMAL	PERCENTAGE OF CALCIUM IN BONES	CALCIUM IN 100 GMS. DRIED TISSUE						
			Heart	Kidneys	Muscle	Liver	Lymph nodes	Lungs	Spleen
	grams		*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	518	20.1	52	21	40	32		51	
2	450	21.8	65	43	48	29	71		38
3	625	23.7	51	53	49	47		43	80
4	575	23.45	47	19	54	40		40	38
5	490	22.34	35	44	36	37	55	30.3	
6	485	24.94	71	48	37	30	25	56	61
7	570	24.98	44	64	57	26	50	65	47
8	470	20.33	43	32	46	28	62	65	20
9	480	24.08	54	58	54	22	65	73	45
10	420	26.67	30.8	33.8	35		60	34	62
11	480		69	16	42	67	71	55.7	58
12	410		52	65	40	60	37	32	46
Average		23.09	51.15	41.40	44.6	38.00	52.8	48.5	49.5

TABLE 2

Calcium determinations of organs of normal guinea pigs fed 0.0338 gm. calcium daily

NUMBER	NUMBER OF DAYS FED CALCIUM	PERCENTAGE OF CALCIUM IN BONES	HEART—MGM. CA. PER 100 GM. DRIED TISSUE	KIDNEYS—MGM. CA. PER 100 GM. DRIED TISSUE	MUSCLE—MGM. CA. PER 100 GM. DRIED TISSUE	LIVER—MGM. CA. PER 100 GM.	LYMPH NODES—MGM. CA. PER 100 GM.	LUNGS—MGM. CA. PER 100 GM.	SPLEEN—MGM. CA. PER 100 GM. DRIED TISSUE
36	15	21.92	58.24	37.8	23.7	53.8	64.5	58.6	83.2
31	27	21.78	83.02	58.5	20.0	35.7	63.0	42.4	47.3
32	41	26.86	103.01	65.3	32.1	17.1	41.7	41.0	41.0
33	56	25.15	61.1	92.0	11.7	47.6	56.7	28.0	44.6
34	87	22.42	46.9	71.6	25.4	17.0	52.0	43.0	64.1
35	95	24.40	46.2	62.6	17.8	39.4	87.2	71.3	47.1
15	182	27.96	79.5	41.5	19.8	36.7	78.2	41.7	70.2
14	213	21.02	63.2	41.0	10.7	23.1	292.8	62.7	40.7
13	214	18.67	64.7	38.5	34.2	56.04	113.0	58.0	36.7
16	228	25.84	40.5	26.8	48.2	38.0			49.3
Average		23.60	64.63	53.56	24.36	36.44	94.3	49.6	52.43

[8] See Mayer and Wells, *The Chemical Composition of Calcified Tuberculous Lesions*, AMER. REV. TUBERC., 1922, vi, 649.

is apparent that the calcium is in equilibrium between the blood and the tissues.

For comparison we have in table 2 the results of analyses of the tissues of ten normal guinea pigs fed calcium for varying periods of time (15 to 228 days). Here we find that the bones, liver, lungs and spleen contain about the same amount of calcium as the unfed controls, the muscles contain less, and the kidneys, lymph nodes and heart contain more calcium. Since the kidney excretes calcium, it is to be expected that its calcium content will be raised by the increased amount of calcium in the urine in the renal tubules, and perhaps in passage through the renal tubules. It has been found by Wells and Hedenburg (45) that ingested iodides are also found in the kidneys in larger amounts than in any other normal tissue. The high figure for the lymph nodes probably depends on deposition of inhaled calcium dust, as mentioned previously, for except for two nodes with very large amounts of calcium the lymph nodes of the calcium-fed guinea pigs contained but little more calcium (62 mgm. per 100 gm.) than in the control guinea pigs. The high figure for the heart depends on one exceptionally high result, which gives rise to the suspicion that this heart may have contained some calcified lesion not noticed at autopsy. Similar instances of occasional high calcium content in the heart have been found in other series of experiments. Disregarding these exceptional instances, we find that administration of calcium to normal guinea pigs has not generally increased appreciably the calcium content of their tissues. No recognizable difference is found between animals that have received calcium for long or for short periods, indicating that in normal animals, at least, there is no tendency for administered calcium to accumulate in the tissues.

In order to secure evidence as to whether there is any retention of calcium in the body as a whole, perhaps not exhibited by the organs and tissues analyzed in these guinea pigs, two series of experiments were carried out with mice, since these animals can readily be analyzed entire.

Twelve mice were fed a mixture of one part whole milk powder (*Dryco* = fat, 12 per cent; lactose, 44 per cent; protein, 34 per cent; salts, 7 per cent; moisture, 3 per cent) and two parts yellow corn meal, being permitted to eat as much as they would.

Twelve mice were fed the same mixture, containing in addition 5 per cent of calcium lactate. The mice were fed this diet for twelve days. On the evening (eight o'clock) of the twelfth day all food was

taken from them and they were starved until nine o'clock of the following day. They were then killed with illuminating gas, skinned, since there might well be adherent dust, and their alimentary tracts removed. The bodies were then weighed without drying.

There was evidence of diarrhoea among both sets of mice, but at autopsy the evidence was much more marked in the calcium-fed mice. The results are given in table 3.

It is seen that the total amount of calcium in the entire ash of the two sets of mice is practically the same (0.1643 gm. as aganst 0.1588 gm.), there being a trifle more in the mice *not* fed calcium. The percentage of calcium in the total fresh weight is, however, a little higher

TABLE 3

MICE NOT FED CALCIUM				MICE FED CALCIUM			
Number	Grams fresh weight of skinned mouse	Grams total calcium in ash	Percentage of calcium	Number	Grams fresh weight of skinned mouse	Grams total calcium in ash	Percentage of calcium
1	9.1668	0.1470	1.60	13	10.2486	0.1443	1.40
2	13.1661	0.1714	1.32	14	10.0837	0.1504	1.49
3	14.2815	0.1647	1.15	15	10.1253	0.1762	1.74
4	13.9001	0.1720	1.23	16	12.0577	0.1551	1.23
6	14.4465	0.1767	1.22	17	12.2373	0.1744	1.42
7	14.2364	0.1668	1.17	18	12.1554	0.1925	1.58
8	13.7274	0.1580	1.15	19	11.2468	0.1946	1.73
9	11.3528	0.1443	1.27	20	8.2940	0.1580	1.90
10	13.7592	0.1615	1.17	21	9.2034	0.0972	1.05
11	11.9963	0.1727	1.40	22	12.4045	0.1685	1.36
12	14.8981	0.1729	1.16	23	10.2681	0.1363	1.32
Averages	13.1755	0.1643	1.25		10.7568	0.1588	1.47

in the mice that were fed calcium (1.25 per cent and 1.47 per cent), which probably depends on their being more dehydrated by the diarrhoea, as indicated by the relatively low weight of these mice.

Because of the complicating influence of the diarrhoea, this experiment was repeated, the diarrhoea being prevented by adding filter paper fragments to the feed. The analytical results are given in table 4.

In this series there was little or no diarrhoea, and here the total calcium in the mice of each series is practically identical (average, 0.1441 and 0.1449 gm.), the percentage of calcium being a trifle higher in the mice not fed calcium, but not beyond the difference possible from the chances of sampling in a series no larger than this.

These experiments corroborate the observations on the guinea pigs in indicating that the administration of relatively large amounts of calcium salts to animals on a normal diet does not increase the amount of calcium in their bodies. Under these conditions the extra calcium is not at all retained.

TABLE 4

MICE NOT FED CALCIUM				MICE FED CALCIUM			
Number	Grams weight of mouse	Grams total calcium	Percentage of calcium	Number	Grams weight of mouse	Grams total calcium	Percentage of calcium
11	12.5365	0.1360	1.09	1	11.4432	0.1284	1.12
12	11.5709	0.1642	1.43	2	12.4160	0.1651	1.32
13	11.0702	0.1482	1.33	3	9.7712	0.1114	1.14
14	8.9281	0.1182	1.31	4	14.2517	0.1565	1.09
15	11.7241	0.1366	1.16	5	12.0084	0.1441	1.18
16	10.5983	0.1579	1.45	6	15.1850	0.1855	1.15
17	9.7325	0.1252	1.31	7	10.3635	0.1459	1.40
18	13.8150	0.1667	1.20	8	12.2848	0.1650	1.34
				9	11.5637	0.1393	1.26
				10	11.7688	0.1280	1.09
Averages	10.4969	0.1441	1.28		12.1056	0.1449	1.209

THE CALCIUM CONTENT OF THE TISSUES OF TUBERCULOUS ANIMALS

We next undertook to ascertain the influence of tuberculosis on the calcium content of the tissues, and, if possible, the influence of calcium administration on the tuberculosis. For this purpose the following experiments were carried out:

Series 1: Twelve guinea pigs, weighing from 508 to 638 gm., were given each a subcutaneous injection of 0.5 mgm. of a strain of human tubercle bacilli of low virulence[9] (no. 1305), on January 31, 1921. One month later the regional lymph nodes were palpable, and on March 3rd 6 of the animals were selected and each given from that time daily, except Sundays, 0.188 gm. calcium lactate (containing 0.0338 gm. Ca) by mouth in gelatin capsules. At the same time 6 normal guinea pigs were started on the same course of calcium feeding. Of this series of 18 animals, 2 died early of pneumonia and a third was discarded as it bore young, leaving five sets of 3 animals each; that is, (1) tuberculosis plus calcium, (2) tuberculosis without calcium and (3) calcium without

[9] We wish to express our obligation to Dr. Lydia M. DeWitt, for the inoculation of the animals used in these experiments.

tuberculosis. All were kept under the same conditions and on the ordinary laboratory diet of oats, hay and carrots. When one of these guinea pigs died, the other two of the set were killed, and the tissues analyzed for calcium.

None of the animals of this series died of tuberculosis, because of the low virulence of the strain of bacillus used. Six of ten showed evidence of tuberculous infection, in the form of enlarged lymph nodes at the site of inoculation, and in two there was a slightly enlarged spleen. One (no. 9) died of pneumonia seventy-four days after inoculation, and one normal calcium-fed guinea pig (no. 18) died of pericarditis and pneumonia. The analytical results obtained with these animals are given in tables 5 and 6 and show no particular differences between the animals which

TABLE 5

Calcium determinations of organs of guinea pigs with chronic tuberculosis and not fed calcium

NUMBER	NUMBER DAYS LIVED AFTER INJECTION	PERCENTAGE OF CALCIUM IN BONES	CALCIUM PER 100 GMS. DRIED TISSUE						
			Heart	Kidneys	Muscle	Liver	Lymph nodes	Lung	Spleen
			mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
4	107	29.32	90.0	53.0	35.9	50.0	48.4	37.5	270.0
3	178	26.11	76.7	43.2	32.4	26.82	252.6	65.4	33.5
2	213	26.75	53.7	40.0	36.1	35.6	98.2	42.2	86.2
5	213	25.84	42.3	59.8	24.9	37.7	54.3	58.5	50.7
6	213	26.67	55.4	73.5	15.4	22.4	40.5	63.4	53.0

Nos. 2 and 6 were very well nourished, and apparently entirely normal. Nos. 3 and 5 had slightly enlarged inguinal lymph nodes. No. 4 had enlarged lymph nodes. Its spleen was not enlarged but showed nodular caseous tuberculosis. Its lungs were apparently normal.

received calcium and the controls. It is to be noted that the only spleen which showed much tuberculosis, that of no. 4, was the spleen with the greatest amount of calcium, and was from an animal which had not been given calcium.

Series 2: Twelve guinea pigs, weighing from 337 to 555 gm., were injected subcutaneously May 4, 1921, with 0.25 mgm. of no. 4015, a highly virulent culture of human tubercle bacilli. Six of these animals died of an acute intercurrent infection, because of which, on May 25th 13 more were given a similar inoculation. On June 8th the local lymph nodes were palpable, and calcium feeding was started as in *series 1* (0.0338 gm. Ca daily) with 6 of these animals.

All animals of this series showed evidences of tuberculous infection at autopsy. Six of them died of tuberculosis (nos. 49, 42, 40, 39, 37,

19), two were killed as they were dying (nos. 38 and 42), while 9 were killed as controls to those that died (nos. *20*, *21*, 28, 30, *43*, *44*, *46*, *47*, *48*). The numbers in italics are those of the guinea pigs that had received calcium, and it will be noted that these in general outlived the animals that had not received calcium. Two animals died of undetermined cause, not tuberculosis (*45* and 41).

The details of these analyses are given in tables 7, 8 and 9.

Consideration of tables 5 to 9 for the tuberculous animals shows that while the organs which do not have tuberculous lesions (heart, kidneys, muscle) contain much the same quantity of calcium as do the same organs of nontuberculous animals, yet in the organs showing the most

TABLE 6

Calcium determinations of organs of guinea pigs with chronic tuberculosis and fed 0.0338 gm. calcium daily

NUMBER	NUMBER DAYS LIVED AFTER INJECTION	NUMBER DAYS FED CALCIUM	PERCENTAGE OF CALCIUM IN BONES	CALCIUM PER 100 GMS. DRIED TISSUE						
				Heart	Kidneys	Muscle	Liver	Lymph nodes	Lung	Spleen
				mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
9	107	74	22.91	49.0	31.0	86.0	46.0	240.0	54.0	68.0
10	178	147	28.17	67.6	46.9	44.3	18.7	68.0	61.1	51.04
11	213	182	23.09	35.4	40.1	28.1	32.1	7.0	50.8	64.5
12	213	182	26.07	63.6	76.8	54.5	30.0	108.9	51.4	19.5
7	213	182	27.04	55.8	59.07	40.0	23.3	20.7	57.5	82.6

No. 9 died of pneumonia: its spleen and lymph nodes appeared normal. Nos. 10 and 11 were killed: their lymph nodes at the site of inoculation were slightly enlarged. No. 12 was killed: on microscopic examination of the spleen numerous typical tubercles of the epithelioid type were seen: the lymph nodes showed extensive fibroplastic tuberculosis. No. 7 was killed: it was apparently normal throughout.

marked lesions (spleen and lymph nodes) the proportion of calcium is greatly increased. This illustrates the tendency of calcium to deposit even in actively progressing tuberculous lesions,[10] but it is to be noted that the amount of calcium in the tuberculous spleens and lymph nodes may be either less or greater in the animals that did not get calcium.

The bones of tuberculous animals, whether fed calcium or not, contain normal quantities of calcium, a finding which fails to support the statement of Sarvonat and Rebattu (based on six animals), that there is a depletion of calcium in the bones.

[10] Caldwell (*J. Infect. Dis.*, 1919, xxiv, 81) found that bovine tuberculous tissues which do not exhibit any macroscopic evidence of calcification often have a very high calcium content.

TABLE 7

Calcium determinations of organs of acutely tuberculous guinea pigs which were not fed calcium

NUMBER	NUMBER DAYS LIVED AFTER INJECTION	PERCENTAGE OF CALCIUM IN BONES	CALCIUM IN 100 GMS. DRIED TISSUE						
			Heart	Kidney	Muscle	Liver	Lymph nodes	Lung	Spleen
			mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
40	54	23.55	61.6	67.1	61.8	25.6	56.2	31.4	39.2
28	55	23.35	113.2	85.6	55.4	160.0	106.2	37.0	137.9
41	65	24.75	55.1	63.3	64.4	20.6	78.2	40.5	143.1
37	65	25.87	54.7	63.7	46.4	20.6	125.5	73.6	673.0*
38	78	24.43	62.9	61.8	12.1	52.8	104.9	37.4	64.9
30	100	25.80	51.4	58.1	36.8		150.9	48.4	39.7
49	105	26.90	76.2	81.5	13.8	7.4	19.0	29.2	3.5
24	107	23.16	44.9	85.5	28.2	24.6	125.9	69.3	26.13
42	131	25.84	77.2	53.9	52.8	16.6	159.08	94.1	119.0*
39	144	25.32	56.9	26.4	9.8	23.1	181.0	129.7	39.9
Averages		24.89	65.41	64.69	38.15	46.8	100.58	59.06	128.6

* The tuberculous portions of the spleens of nos. 37 and 42 were analyzed separately from the nontuberculous portions. Calcium estimations were: no. 37, tuberculous portion, 2027.8 mgm., nontuberculous portion, 250.6 mgm.; no. 42, tuberculous portion, 143.6 mgm., nontuberculous portion, 59.0 mgm.

No. 40: Died of tuberculous pneumonia. Histological examination showed a few tubercles in lung; in spleen many active miliary tubercles, comprising about 30 per cent of tissue of spleen. The lymph nodes moderately enlarged.

No. 28: Killed as control. Histological examination of liver revealed numerous tubercles; the lung numerous typical tubercles; the spleen had more tubercles than normal tissue. A parapancreatic lymph node contained large tubercles and small focal tubercles. Macroscopically, the lymph nodes were much enlarged, and some were caseous.

No. 41: Died. Spleen studded with miliary tubercles. Histologically, lungs showed no tubercles, but some edema. The liver was considerably enlarged and contained a few superficial necrotic areas. Lymph nodes only slightly enlarged.

No. 37: Died. Histological examination of the lungs showed that over 50 per cent had been replaced by tuberculous tissue. In the liver there was much fibrosis, as in repair of recent necrotic areas, but there was no distinct tuberculosis. The spleen contained numerous tubercles and some necrotic areas. The lymph glands were largely tuberculous.

No. 38: Killed as control. Histological examination. The lung tissue was largely replaced by consolidated areas, not definitely tuberculous. In the spleen, which was enlarged, there were no distinct tubercles. The liver had necrotic areas, but no tubercles.

No. 30: Killed as control. Histological examination: In the spleen and liver, a very few miliary tubercles. The lungs contained very many tubercles and much edema. A peritracheal lymph node was largely replaced by tuberculosis. The inguinal and peribronchial lymph nodes were much enlarged and caseous.

No. 49: Died. Histological examination of the liver showed it to be fatty, but no tubercles were seen. In the lungs there were many miliary tubercles, amounting to about 10 per cent of the lung tissue. The spleen was greatly congested with some necrosis, but no typical tubercles were seen. One inguinal lymph node was slightly enlarged and caseous.

No. 24: Killed as it was dying. The lymph nodes in inguinal region were much enlarged and caseous. Histologically, the spleen was seen to be riddled with active tubercles, comprising about 25 per cent of the spleen tissue. In the lungs there were occasional active tubercles. The liver was apparently normal.

No. 42: Died. The inguinal and peribronchial lymph nodes were enlarged and caseous. Histological examination of the spleen showed that it was largely necrotic, with areas resembling infarcts, which were not typical tubercles. The lungs showed numerous large acute tubercles. The liver contained many areas of fibrosis and only a few active tubercles.

No. 39: Died. Histological examination of a lymph node showed many small, rather fibrous tubercles, and large necrotic areas, some of which were distinctly calcified. The liver contained much connective tissue formation and fatty change, but no tubercles. The lung showed large masses of consolidation resembling infarcts, probably tuberculosis. The spleen contained many acute tubercles.

TABLE 8

Calcium determinations on organs of acutely tuberculous guinea pigs which were fed calcium

NUMBER	NUMBER DAYS LIVED AFTER INJECTION	NUMBER DAYS FED CALCIUM	PERCENTAGE OF CALCIUM IN BONES	CALCIUM IN 100 GMS. DRIED TISSUE						
				Heart	Kidneys	Muscle	Liver	Lymph nodes	Lung	Spleen
				mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
47	54	41	24.32	67.0	60.5	14.0	39.2	32.0	91.3	91.6
19	55	20	15.27	80.3	6.0	88.8	38.0	1298.0	60.1	22.1
48	65	50	23.26	49.8	81.6	37.4	33.0	67.6	38.1	50.8
45	69	19	23.19	160.2	36.2	67.3	317.8	301.0	80.9	136.0
46	78	65	25.52	62.5	71.4	37.4	19.6	47.0	60.4	104.4
20	100	64	26.36	86.4	80.0	48.1	70.7	257.8	51.7	190.5
43	131	87	25.65	86.4	58.9	65.00	22.8	132.6	27.0	26.0
21	133	95	25.80	67.7	53.1	37.07	20.3	111.4	38.3	278.0
44	144	131	24.82	44.4	47.5	20.5	16.0	73.3	43.2	61.7
Averages.....			23.78	78.3	55.02	46.17	64.15	257.8	54.5	106.78

No. 47: Killed as control. The inguinal and peribronchial nodes were enlarged, and some were caseous. On histological examination it was found that about 50 per cent of the spleen was made up of tubercles. The lungs showed focal fibrous areas, with occasional distinct tubercles. No tubercles were seen in the liver.

No. 19: Died of pneumonia. The lymph nodes were very much enlarged and caseous. Microscopic examination of the spleen showed that about 60 per cent of it was replaced by epithelioid tubercles. The liver had infarcted areas, some necrotic areas and some fatty change. The lungs had large pneumonic areas, not definitely tuberculous.

No. 48: Killed as control. The inguinal and peribronchial nodes were enlarged. Microscopically, 25 per cent of the spleen was replaced by many small tubercles. The liver contained a few tubercles with a moderate degree of perilobular fibrosis. The lungs had a very few miliary tubercles.

No. 45: Died. The lymph nodes were only slightly enlarged and caseous. The spleen was studded with miliary tubercles. The liver showed a few small necrotic areas only. Lungs showed no visible tuberculosis. Postmortem degeneration too far advanced to tell cause of death.

No. 46: The lymph node in left inguinal region, the site of inoculation, was slightly enlarged and caseous. Microscopically, this node was seen to have occasional epithelioid tubercles. The spleen and liver were apparently normal. The lungs had occasional small tubercles.

No. 20: Killed. The inguinal and peribronchial nodes were much enlarged. They showed, microscopically, much fibrosis with a few active tubercles. The spleen was only moderately enlarged, but covered with nodular tubercles. The liver, microscopically, had a few fibrous tubercles. The lung had numerous tubercles, mostly fibrous.

No. 43: Killed. The peribronchial lymph nodes were slightly enlarged. Microscopic examination of the lungs showed numerous tubercles. The spleen had numerous small active tubercles. The liver showed occasional small tubercles, and much fatty change.

No. 21: Killed. The lymph nodes in the inguinal and peribronchial regions were decidedly enlarged and caseous. The spleen was much enlarged and covered with numerous tubercles. The liver had a few small tubercles. There were a few tubercles visible in the lungs.

No. 44: Killed. At autopsy the only evidences of tuberculosis consisted of one enlarged caseous lymph node at the site of injection and much enlarged peribronchial nodes. Microscopically, these nodes showed extensive fibroplastic tuberculosis. The lungs were edematous but no tubercles were seen.

For some reason the lung, even when extensively tuberculous, is much less likely to show a high calcium figure than either the liver or the spleen. It is to be noted, if the anatomic findings are compared with the chemical findings, that there are many exceptions to the rule that the more tuberculosis an organ shows the more calcium it contains.

The calcium determinations of the lymph nodes and spleens of 18 tuberculous animals, regardless of presence of gross lesions in the spleen (9 fed calcium and 9 not fed calcium), show that in 5 out of 9 pairs of animals, the calcium content of the lymph nodes of the calcium-fed guinea pigs was less than that of the guinea pigs which were not fed calcium. In 5 out of 9 pairs of animals the calcium content of the spleens of the calcium-fed guinea pigs was greater than that of the animals not fed calcium.

However, the extent of the tuberculous lesions was much greater in the lymph nodes in 7 out of 9 cases in the control animals, and in the spleens, in every case, the extent of the tuberculosis was much greater in the control than in the calcium-fed guinea pigs. The average calcium content of the lymph nodes of the calcium-fed animals was 257.8 mgm. per 100 gm. dried tissue, as against 100.58 in the guinea pigs not fed calcium, and the average calcium content of the spleens of the calcium-fed pigs was 106.78 mgm. as against 128.6 in those not fed calcium.

It is somewhat difficult to evaluate these average figures, however, since they are made up of single estimations with such great variations. Compare, for example, spleen no. 37 with an average of 673 mgm. cal-

TABLE 9

General summary of acutely tuberculous guinea pigs

	PERCENTAGE OF CALCIUM IN BONES	CALCIUM IN 100 GMS. DRIED TISSUE						
		Heart	Kidneys	Muscle	Liver	Lymph nodes	Lungs	Spleen
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
Averages for all (20) calcium-fed guinea pigs, with or without tuberculosis	23.32	71.10	54.25	34.69	55.86	176.05	52.06	78.18
Min.-Max.	15.27–27.96	40.5–160.0	6.0–92.0	10.7–88.0	16.0–317.8	32.0–1298	27.0–91.3	22.1–278.0
Average for (9) calcium-fed tuberculous guinea pigs	23.78	78.3	55.02	46.17	64.15	257.8	54.5	106.78
Min.-Max.	15.27–26.36	44.0–160.0	6.0–81.6	14.0–88.0	16.0–317.8	32.0–1298	27.0–91.3	22.1–278.0
Average for (11) calcium-fed guinea pigs, not tuberculous	23.85	64.57	53.56	24.33	36.44	94.3	49.6	52.43
Min.-Max.	18.67–27.96	40.5–103.0	26.8–92.0	10.7–48.2	16.0–317.8	32.0–1298.0	27.0–91.3	22.1–278.0
Average for (10) tuberculous guinea pigs, not fed calcium	24.89	65.41	64.69	38.15	46.8	100.58	59.06	128.6
Min.-Max.	23.16–26.90	44.9–113.2	26.4–85.6	9.8–64.4	7.4–160.0	19.0–181.0	29.2–129.7	3.5–673.0
Average for (12) guinea pigs not fed calcium and not tuberculous	23.09	51.15	41.40	44.6	38.00	52.8	48.5	49.5
Min.-Max.	20.1–26.67	30.8–71.0	16.0–65.0	35.0–57.0	26.0–67.0	25.0–71.0	30.0–73.0	20.0–80.0

TABLE 10

Relation of calcium deposition to tuberculous lesions in acutely tuberculous guinea pigs

	CALCIUM IN 100 GMS. DRIED TISSUE			
	Liver	Lymph nodes	Lungs	Spleen
	mgm.	*mgm.*	*mgm.*	*mgm.*
Averages for all tuberculous guinea pigs	55.4	175.05	56.89	118.2
Min.-Max.	16.0–317.8	19.0–1298.0	27.0–129.7	3.5–673.0
Averages for tuberculous guinea pigs with lesions in tissues listed	59.16	175.05	57.31	112.7
Min.-Max.	7.4–317.8	19.0–1298.0	27.0–129.7	3.5–673.0
Averages for tuberculous guinea pigs with lesions; not fed calcium	43.0	100.58	61.1	128.6
Min.-Max.	7.4–160.0	19.0–181.0	29.2–129.7	3.5–673.0
Averages for tuberculous guinea pigs with lesions; fed calcium	83.6	257.8	52.4	106.07
Min.-Max.	20.3–317.0	32.0–1298.0	27.0–91.3	22.1–278.0
Averages for tuberculous guinea pigs without lesions in tissues listed	25.0	All tuberculous guinea pigs have some enlarged lymph nodes	54.8	104.4
Min.-Max.	16.0–39.2		40.5–80.9	One guinea pig
Averages for tuberculous guinea pigs without lesions; fed calcium	24.9	All tuberculous guinea pigs have some enlarged lymph nodes	62.05	104.4
Min.-Max.	16.0–39.2		43.2–80.9	One guinea pig
Averages for tuberculous guinea pigs without lesions; not fed calcium	25.1	All tuberculous guinea pigs have some enlarged lymph nodes	40.5	Lesions in all guinea pigs not fed calcium
Min.-Max.	24.6–25.6		One guinea pig	

cium per 100 gm. and no. 49 with but 3.5 mgm. Much of this difference depends on the degree of tuberculous involvement, as shown in table 10, this seeming to have a far greater influence on the calcium content of the tissues than the administration of calcium.

Hence these experiments do *not* indicate that administration of large amounts of calcium causes larger amounts to be deposited in tuberculous lesions than will be the case in an animal on a normal diet which contains ordinarily adequate amounts of calcium.

TUBERCULOSIS OF THE TESTICLE

As it was not possible to separate cleanly tuberculous and nontuberculous portions of the same organ from one another in guinea pigs with systemic disease, in order to compare more accurately the influence of the tuberculous focus itself upon the calcium content of the tissue, attempts were made to secure tuberculosis of one of a pair of organs and to compare its calcium content with that of the normal mate. Doctor DeWitt first inoculated a series of guinea pigs directly into one lung, but it was not found possible in this way to secure a lung with extensive tuberculosis while its mate was normal or nearly so. Much greater success was obtained when the inoculation was made directly into one testicle. Although guinea pigs with systemic tuberculosis do not often exhibit extensive lesions in the testicle, this organ was found to be very susceptible to direct inoculation. Occasionally, a few small lesions were found in the uninoculated testicle, but these were not sufficient to affect the value of the experiment. This experiment was conducted as follows:

Fourteen animals were inoculated April 5, 1922. Each received 0.1 mgm., culture no. 4015, in the left testis. Seven were fed calcium daily from the day of inoculation. In all animals (except one) the inoculated testicle showed more evidence of tuberculous infection than its control. The exception was no. 82. Two small tuberculous nodules were visible in the noninoculated testis of no. 80. Two animals, nos. 79A and 79B, were much larger animals and were given 8 mgm. of the same culture. The animals were killed at the end of periods varying from 15 to 63 days, but the amount of involvement was by no means proportional to the duration of the infection. The results are shown in table 11.

Examination of this table reveals that in all cases the tuberculous testicle was larger than its mate, and with few exceptions contained

more calcium per unit of weight. This illustrates again the fact that tuberculous lesions take on calcium even when there is neither gross nor microscopic evidence of calcification, a property which seems to be common to necrotic tissues of whatever sort. This same difference is well shown in the two spleens that exhibited lesions which could be separated from the relatively normal tissue (table 7, nos. 37 and 42). Here the tuberculous portion contained very much more calcium than the nontuberculous portions of the same organ.

It so happens that the proportion of calcium averages somewhat higher in the testicles of guinea pigs not fed calcium than in those that did receive calcium. This, however, has little significance in so short a series, since in each group there is one specimen that has many times more

TABLE 11

GUINEA PIGS NOT FED CALCIUM						GUINEA PIGS FED CALCIUM					
Number	Length of experiment in days	Tuberculous testicle		Nontuberculous testicle		Number	Length of experiment in days	Tuberculous testicle		Nontuberculous testicle	
		Weight of dried testicle	Calcium mgm. in 100 gm.	Weight of dried testicle	Calcium mgm. in 100 gm.			Weight of dried testicle	Calcium mgm. in 100 gm.	Weight of dried testicle	Calcium mgm. in 100 gm.
92	45	0.2983	96	0.1985	67	79A	15	1.1853	1064	0.5268	37
89	46	0.3295	43	0.0543	47	79B	23	0.4523	41	0.2854	18
91	46	0.5176	1225	0.0897	504	85	31	0.2255	209	0.0640	120
93	47	0.2533	238	0.0672	20	84	45	0.2009	104	0.1345	126
90	49	0.1705	79.1	0.0693	47	82	49	0.4147	142	0.2154	64
88	51	0.2127	95	0.0536	52	81	49	0.1817	143	0.0747	100
87	55	0.1714	392	0.0523	52	83	53	0.1351	265	0.0527	100
						86	53	0.1991	209	0.1136	120
						80	63	0.3400	40	0.0695	120
Average		0.2647	309	0.0835	112			0.3483	246	0.1707	89

calcium than any of the others. If these are eliminated, the averages are not very different from one another. It will be noticed that in a few instances the nontuberculous testicle has the greater proportion of calcium; perhaps this depends on the existence in the tuberculous testicle of an early cellular stage of lesion without any necrotic tissue to increase the calcium content. However, we did not find the amount of calcium to vary directly with the amount of caseation visible macroscopically.

It has been found (46) that as little as 2 mgm. of calcium in an entire rabbit kidney may be detected microscopically as fine, dust-like particles, and 5 mgm. constitute a noteworthy microscopic calcification. This would be a proportion of about 100 mgm. of calcium per 100 gm. dry weight.

THE INFLUENCE OF CALCIUM ON THE COURSE OF EXPERIMENTAL TUBERCULOSIS

In some of the earlier experiments the impression was obtained that the tuberculous animals that received calcium did somewhat better than the controls, but whatever differences were observed were not constant or sufficiently marked to be convincing. In order to see whether any influence of calcium administration could be discerned in tuberculous guinea pigs, the following experiment was performed:

Twenty-four guinea pigs were inoculated subcutaneously with 0.005 mgm. of culture no. 4105. Twelve of these were fed calcium from the day of inoculation, while twelve were not fed calcium. Eighteen of these animals, evenly divided between calcium-fed and controls, died within 135 days. The lymph nodes, spleens, lungs, and livers of the

TABLE 12

Analyses of organs of calcium-fed and non-calcium-fed tuberculous animals

	CALCIUM IN 100 GMS. DRIED SUBSTANCE	
	Organs of guinea pigs *not* fed calcium	Organs of guinea pigs fed calcium
	mgm.	*mgm.*
Lungs (9)	206	107
Lymph nodes (9)	339	715
Spleens (9)	313	128
Livers (8)	59	62

treated and untreated animals were united and analyzed in two separate lots for calcium, so that each figure represents actually the average of eight or nine organs, without consideration of the degree of tuberculous involvement (table 12).

The results agree well with those obtained previously (tables 5 to 10), in showing that the highest calcium figures are given by the lymph nodes, which likewise have the most extensive tuberculosis. The spleen, with the next largest amount of tuberculosis, comes next, closely followed by the lung, while the liver shows relatively little calcium, in correspondence with its relative lack of tuberculous lesions. Here again the lymph nodes of the animals fed calcium show the highest calcium content, but as the liver and spleen exhibit the opposite relation it is probable that this excess in the lymph nodes comes from inhaled dust.

The average length of life of the calcium-fed guinea pigs was 92.5 days, while that of those not fed calcium was 74.4 days. Such a slight

difference is of no significance whatever, for nothing is less constant than the length of life of tuberculous guinea pigs even under identical conditions of dosage and environment.

In two of the animals which were not fed calcium the only evidence of tuberculous infection was found in an enlarged lymph node in the left groin, the site of inoculation. The lymph nodes, lungs and spleen of the 7 remaining guinea pigs showed marked progress of the infection. In the calcium-fed series one animal died of an unknown cause. The other 8 animals showed a severe generalized tuberculosis.

The livers in both series of animals were involved in 8 cases. If there was any difference in the degree of involvement of the organs, it was more marked in the animals that had been fed calcium.

A study of their weight curves (chart 1)[11] shows no essential difference in the two sets of animals. The observations of DeWitt (47) on the weight curves in tuberculous guinea pigs have indicated that these may be of some value in estimating the influence of treatment on the disease.

A study was made of the microscopic features of the lesions in the animals of this series, with particular reference to the degree of fibroplastic reaction to the tuberculous infection. Although this is to some extent determined by the length of time the animals lived, it was found that, when the sections were classified according to the amount of fibrosis in and about the lesions, somewhat more fibrotic lesions were found in the animals not fed calcium. Also, a few more foci showing microscopically visible calcification were found among the lesions from the animals that were not fed calcium, although the average life in this series was somewhat shorter. At least it can be said with certainty that in this experiment the administration of calcium has not reduced the amount of spread of the lesion nor has it led to a greater amount of fibroplastic tissue reaction.

In addition to the eighteen guinea pigs of this series described above there were six others that lived more than eight months. It so happened that three of these were animals that were being fed calcium and three which had no calcium. Here again the gross and histological appearances in the two groups of three animals each, showed no noteworthy differences except that as before there was, if anything, more fibrosis in the three animals that had not been fed calcium. Only one animal

[11] The numbers on the curves in this and succeeding charts indicate the number of animals furnishing the figures at this date.

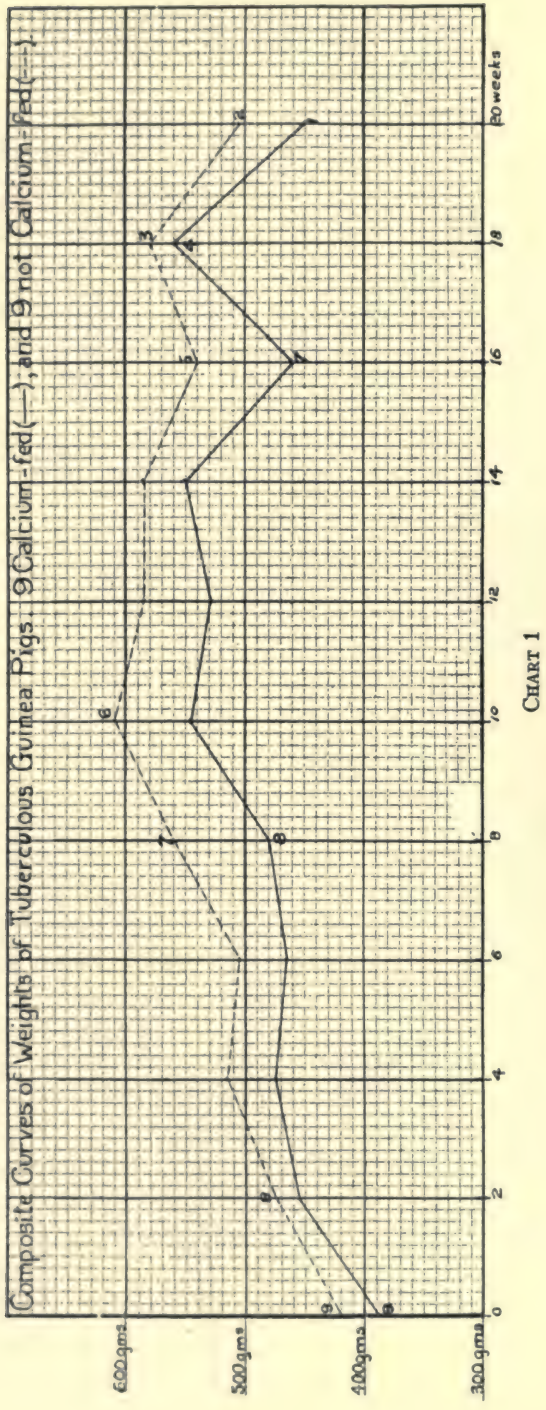
Composite Curves of Weights of Tuberculous Guinea Pigs. 9 Calcium fed (—), and 9 not Calcium fed (---).
600 gms
500 gms
400 gms
300 gms
0
2
4
6
8
10
12
14
16
18
20 weeks

CHART 1

showed calcific deposits visible under the microscope and this animal (no. 61) was one that had not been given calcium; there were large calcified areas in the spleen and a few small calcified areas in the lymph glands and lungs. The organs of the three animals of each series were analyzed together and the results are given in table 13. Corresponding to the microscopic findings, we have distinctly more deposition of calcium in the tissues of the animals that did not receive calcium.

TABLE 13

The calcium content of the organs of guinea pigs that lived longer than eight months after inoculation

	CALCIUM IN 100 GMS. DRIED TISSUE			
	Lungs	Lymph nodes	Spleens	Livers
	mgm.	*mgm.*	*mgm.*	*mgm.*
Calcium fed tuberculous, 62, 67, 71.....	174.4	450.2	233	75.0
Tuberculous, no calcium treatment, 56, 57, 61 ..	193.8	510.9	388	74.1

BLOOD CHANGES UNDER CALCIUM FEEDING

Having under observation a series of animals with and without tuberculosis, some of which were receiving calcium and some not, a study of the blood changes was considered worth making, especially because in this laboratory Dr. DeWitt and Miss Lauretta Bender have been carrying on an extensive study of the blood in tuberculous guinea pigs, a preliminary report of which was given at the 1922 meeting of the National Tuberculosis Association.

Chart 2 shows the coagulation time of the blood of these animals, and reveals that, contrary to expectation, the animals fed calcium showed a slower coagulation of the blood than the normal guinea pigs. While the tuberculous animals without calcium showed a much slower coagulation time than the normals, the calcium-fed guinea pigs with tuberculosis had a more rapid coagulation time than the tuberculous ones without calcium. The differences were, however, scarcely great enough to be of much significance. It may be recalled that in human tuberculosis the coagulation time has generally been found to be somewhat more rapid than normal (48).

Systematic blood counts were made and the results are shown in chart 3. It will be seen that the blood count was little altered as the tuberculosis progressed in these guinea pigs and that there was no noteworthy

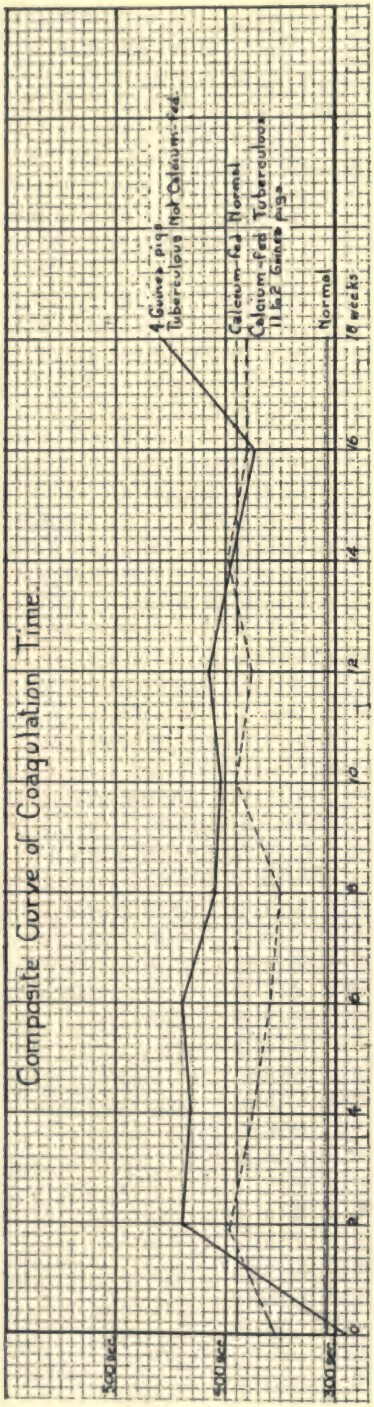
Composite Curve of Coagulation Time
4 Guinea Pigs Tuberculous Not Calcium-fed.
Calcium-fed Normal
Calcium-fed Tuberculous 11 to 2 Grams Pigs
Normal
18 weeks
16
14
12
10
8
6
4
2
0
500 sec.
500 sec.
500 sec.

CHART 2

difference between the calcium-fed guinea pigs and those that did not receive calcium. Only the hemoglobin was altered. It ran steadily between 58 and 64 per cent in the animals fed calcium, but in those not fed calcium it started at 74 per cent and fell steadily to about 60 per cent, after which it continued at about the same level as in the calcium-

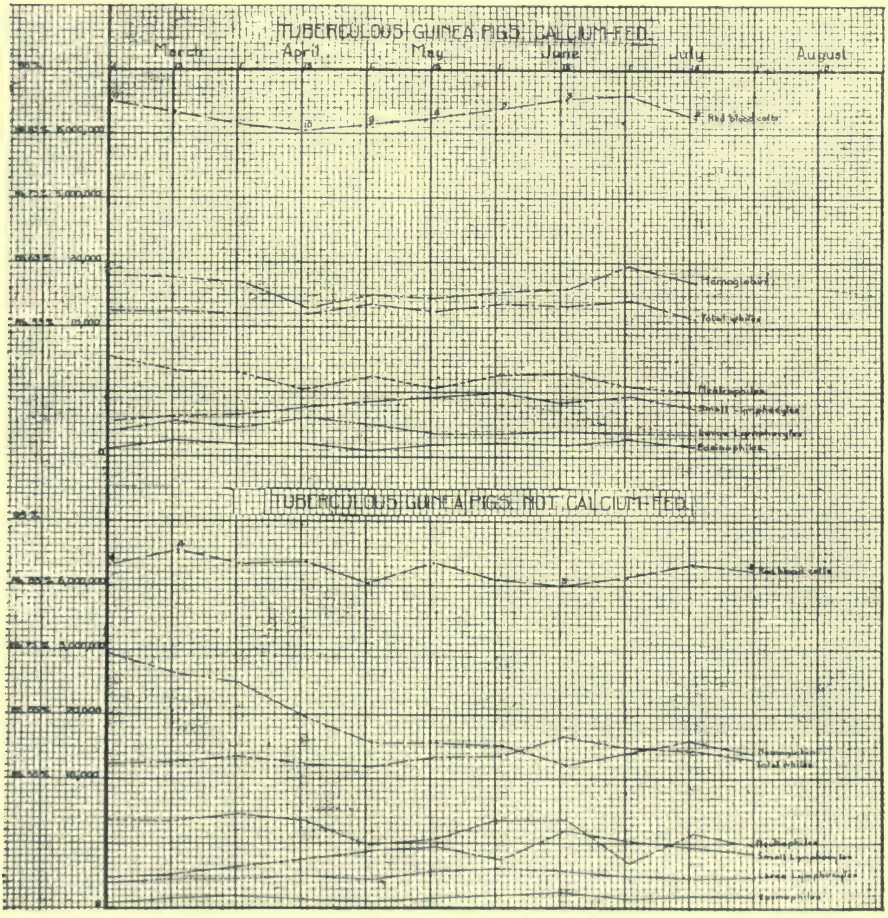

CHART 3

fed animals. This variation in the hemoglobin was not accompanied by any corresponding change in the number of red cells, which remained nearly constant. Usually the hemoglobin curve in guinea pigs has been found to start at a higher level, that is, from 80 to 90 per cent, and to fall in the acutely tuberculous guinea pigs somewhat more than the red cell count, as it did in these animals.

SUMMARY

A review of the literature on the therapeutic use of calcium in tuberculosis, and especially the experimental evidence on this subject, reveals the presence of a considerable body of clinical observation indicating a favorable effect of calcium therapy upon the tuberculosis itself as well as on certain of its complications, a certain amount of evidence that such favorable results are not obtainable, and a complete lack of convincing experimental evidence in support of calcium therapy. The hypothesis of a decalcification or demineralization in tuberculosis does not receive support from the most carefully conducted investigations. Also, the best chemical evidence is that there is no demonstrable deficiency of calcium in the blood or tissues in tuberculosis, and that feeding of calcium salts does not appreciably increase the amount of calcium in the blood when this is already normal.

Our own experiments have shown that guinea pigs on an ordinary laboratory diet exhibit some variations in the calcium content of the same tissue in different animals, and of different tissues in the same animals, yet, averaged together in a series of animals, the results are not far from the same, being generally lowest in the liver and highest in the lymph nodes, but not far from the same content as the blood, that is, 10 to 13 mgm. of calcium per 100 gm. moist weight.

Addition of calcium lactate to the diet of normal guinea pigs does not increase the amount of calcium to be recovered from most of the tissues. The kidneys may contain a little more, presumably because they are excreting any excess absorbed, and the lymph nodes usually show an increase, presumably from inhalation of dust.

Analysis of the entire bodies of mice (skin and intestines excluded) showed no more calcium in those that had been fed calcium lactate than in those on the standard diet.

Analysis of the tissues of guinea pigs with experimental tuberculosis shows a marked tendency to the accumulation of calcium in the tuberculous tissues, even when calcification is not visible microscopically. The amount of calcium deposition does not always vary in proportion to the extent of the tuberculous change in an organ. It was also found that the amount of calcium in the tuberculous lesions is not appreciably modified by feeding calcium lactate in addition to the usual diet. Sometimes the tuberculous tissues showed more calcium in the animals that received calcium and sometimes there was more in the control animals.

The tuberculous lungs show much less tendency to accumulate calcium than tuberculous spleens, livers or lymph nodes.

Direct inoculation of the testicle in the guinea pig was found to produce a marked involvement of the testicle, furnishing material that could be compared with the normal organ of the same animal. Here again there was a marked deposition of calcium in the tuberculous tissues, but not greater in the animals fed calcium than in the controls.

No demonstrable protective or curative effects could be observed in a limited number of tuberculous guinea pigs fed calcium as compared with the course of the disease in the control animals. There was no evidence that fibroplastic protective or healing reactions were increased by the feeding of calcium.

The blood cytology was not different in a series of calcium-fed tuberculous guinea pigs than in the control animals. The coagulation time in the calcium-fed guinea pigs was somewhat longer than in controls, but it was still longer in the tuberculous guinea pigs that were not fed calcium.

These studies, therefore, have failed to produce any experimental evidence which supports the empirical clinical evidence that calcium administration has a favorable influence upon the course of tuberculous infection. They do support the evidence of chemical studies that administration of calcium salts by mouth does not appreciably increase the amount of calcium in the blood and tissues, for this is normally maintained at a level not far below the saturation point of the body fluids.

REFERENCES

(1) Wersen: Upsala Läkaref. Förh., 1914, xx, 1.
(2) Reckzeh: Berl. klin. Wchnschr., 1903, xl, 1922.
Selkirk: Brit. M. J., November 14, 1908.
Fisac: Siglo Med., Madrid, 1916, lxiii, 717.
Tweddell: Med. Rec., 1922, ci, 141.
(3) Sergent: Presse Méd., 1910, xviii, 865.
(4) Loeper and Béchamp: Compt. rend. Soc. d. Biol., 1909, lxvii, 350; and Ibid., 1910, lxix, 112.
(5) Croftan: N. York M. J., 1909, lxxxix, 1182.
(6) Ott: Chem. Path. d. Tuberk., p. 357.
(7) Mayer, A.: Deutsches Arch. f. klin. Med., 1907, xc, 408.
(8) Vannini: Compt. rend. Soc. d. Biol., 1913, lxxii, 876.
(9) Labbé and Gallippe: Bull. d. sc. med. Bologna, 1908, viii, 341.
(10) Voorhoeve: Deutsches Arch. f. klin. Med., 1913, cx, 231.
(11) McCann: Amer. Rev. Tuberc., 1922, v, 870.
(12) Kiehnle, E.: Führt die Tuberkulose in ihrem Verlauf zu einer vermehrten Caries-frequens, Dissertation, Erlangen, 1921.

(13) SARVONAT AND REBATTU: Compt. rend. Soc. d. Biol., 1910, lxix, 127; and J. Physiol. et Path. Gén., 1910, xii, 934.
(14) KAHN, M.: Med. Rec., May 23, 1914.
(15) LOEPER AND BÉCHAMP: Compt. rend. Soc. de biol., 1910, lxix, 178.
(16) BRACCI: Clin. Mod., 1903, ix, 452.
(17) STEINITZ AND WEIGERT: Deutsche med. Wchnschr., 1904, xxx, 838; and Jahrb. f. Kinderhk., 1905, lxi, 147.
(18) DENIS AND MINOT: J. Biol. Chem., 1920, xli, 357.
(19) GIVENS: Ibid., 1918, xxxiv, 119.
(20) MASON: Ibid., 1921, xlvii, 3.
(21) VOORHOEVE: Biochem. Ztschr., 1911, xxxii, 394.
(22) TOWLES: Am. J. M. Sc., 1910, cxl, 100.
(23) KOST, A.: Inaug. Diss., Bonn, 1913.
(24) HOWLAND AND MARRIOTT: Quart. J. Med., 1918, xi, 289.
(25) JACOBOWITZ: Jahrb. f. Kinderhk., 1920, xcii, 25.
(26) MUGGIA: Riforma Med., 1921, xxxvii, 1050.
(27) HEUBNER AND RONA: Biochem. Ztschr., 1919, xciii, 187.
(28) HALVERSON: J. Am. M. Ass., 1917, lxviii, 1309.
(29) WELLS: Arch. Int. Med., 1911, vii, 721.
(30) VAN GIESON AND LYNAH: Med. Rec., 1912, lxxxii, 883.
(31) VAN GIESON: Nat. Tuberc. Ass. Tr., 1912, viii, 466.
(32) RUSSELL: Med. Rec., 1909, lxxvi, 889 and 1021.
(33) EMMERICH AND LOEW: Arch. f. Hyg., 1913, lxxx, 261.
(34) MASSINI: Schweiz. med. Wchnschr., 1921, li, 223.
(35) MICHELAZZI: Gazz. d. Osp., Milano, 1904; and abstract in Rev. d. l. Tuberc., 1904, i, 421.
(36) LAITINEN, T.: Reference in Zentralbl. f. Tuberk., 1922, xvii, 317.
(37) LEO: Berl. klin. Wchnschr., 1911, xlviii, 1800.
(38) FINSTERWALDER, C.: Arch. f. d. ges. Physiol., Bonn, 1913, cliii, 546.
(39) TAKENAKA: Kekkaku Zassi, 1918, i, no. 2; and Ibid., 1920, iii, no. 6.
(40) WOLF, E. P.: J. Exper. Med., 1921, xxxiv, 375.
(41) RÖSLER: Wien. Arch. inn. Med., 1921, ii, 281.
(42) OPITZ: Deutsche med. Wchnschr., 1920, xlvi, 1391.
(43) KAHN, M.: Med. Rec., May 23, 1914.
(44) KRAMER AND TISDALL: Johns Hopkins Hosp. Bull., 1921, xxxii, 44.
(45) WELLS AND HEDENBURG: J. Infect. Dis., 1912, xi, 349.
(46) WELLS, HOLMES AND HENRY: J. Med. Research, 1911, xxv, 373.
(47) DEWITT: J. Infect. Dis., 1920, xxvii, 503.
(48) SOLIS-COHEN: Arch. Int. Med., 1911, viii, 684; 820.

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STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS

XXV. TISSUE CHANGES PRODUCED BY THE ACTION OF THE LIPINS[1] OF TUBERCLE, GRASS AND COLON BACILLI, AND OF LIVER

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Auclair (1) reported that he had produced caseation-necrosis in guinea pigs and rabbits by subcutaneous and intratracheal injections of lipoidal materials, extracted from human tubercle bacilli with ether and chloroform. In a later publication (2) he referred to his experiments with ether and chloroform extracts, and stated that xylene and benzene extractions yielded substances with similar capacity to produce caseation. In a still later work (3) he stated that he produced a typical caseous pneumonia in the lungs of a rabbit by intratracheal injections of the ether extract of tubercle bacilli.

Morse and Stott (4) produced a fibroblastic reaction of the epithelioid type with numerous typical cellular tubercles and characteristic giant cells in rabbits, by means of subcutaneous, intraperitoneal and intravenous injections of the alcohol extract of tubercle bacilli. The ether extract they found to be inert. In no case did they observe caseation. They concluded that the tubercle bacillus produces a characteristic microscopic lesion, not because it is a living particulate protein but because its waxy substances act as a peculiar type of foreign body.

As far as we can determine there has been no experimental work done on the tissue changes produced by the lipins of nonpathogenic microorganisms. Gaehlinger and Tilmant (5) made experiments with "liver lipoids," and claimed that there exist in the liver lipoids some acid-resisting fatty acids capable of causing tubercles with caseation when these lipins are injected subcutaneously. This property suggests that

[1] In this article the word *lipins* is used in its comprehensive sense to include simple fats, lipoids, fatty acids and related substances.

the hepatic lipins must be similar to the caseifying poison extracted from tubercle bacilli by Auclair, which he designated as *ethero-bacilline*.

We have attempted to repeat the work of Gaehlinger and Tilmant and, in addition, have made an effort to determine the tissue reactions to similar extracts of grass bacilli and colon bacilli, and to compare them with the reactions to corresponding preparations from tubercle bacilli.

PREPARATION OF MATERIALS

Two methods of extracting the lipoids from the bacilli were used. One is essentially similar to that employed by Morse and Stott, except that after the hot alcohol extraction a hot chloroform extraction was made; the other method is different, and was suggested by Dr. E. R. Long. In subsequent references we shall designate the first method as the Morse method, and the second as the Long method.

Morse's Method: Fifty grams of dry tubercle bacilli were put into a flask, 1.5 litres of ether were added, and the flask allowed to remain in a refrigerator over night. The ether was then poured off, passed through a hard filter paper, then through a fine Berkefeld filter, and evaporated practically to dryness and stored in sterile bottles. To the bacilli remaining in the flask 1.5 litres of 95 per cent alcohol were added, and the contents boiled under a reflux condenser for two hours. The alcohol was poured off, filtered hot through a hard filter paper, then through a Berkefeld filter, and evaporated and stored as was the ether extract. To the bacilli remaining in the flask was added 1.5 litres of chloroform, and an extraction made exactly as with the alcohol. The ether-extracted lipin was waxy, solid and dark red. The alcohol-extracted lipin was like semiliquid honey, with at first a honey-like odor, which changed on standing to an indescribable "musty" odor. The chloroform-extracted lipin was a glassy, waxy material, caramel colored.

Long's Method: Three hundred grams of dried tubercle bacilli were put into a large flask with three litres of 95 per cent alcohol and allowed to stand for three days. The alcohol was then poured off, distilled to about 400 cc. volume, filtered through a hard filter paper and then through a Berkefeld filter,[2] poured into a large evaporating dish, evaporated almost to dryness on a water bath, and the dish set aside as a

[2] The passage through the Berkefeld filter was to remove any solid bacillary substance, for extracts merely filtered through paper commonly contain many dead bacilli, which might possibly be responsible for the tissue reactions observed.

receptacle for the hot alcohol extractions which followed. As before, three litres of 95 per cent alcohol were poured into the flask, which was then boiled under a reflux condenser for two hours, and the alcohol treated as before. Then two extractions were made in the same manner with absolute alcohol, the same evaporating dish being used in the final concentration in each case. The bacilli remaining in the flask were then subjected to two hot toluene extractions, using three litres in each extraction, and employing exactly similar technique, another evaporating dish, however, being used for the final concentrations.

Thus we had one large evaporating dish containing all the lipins extracted by the alcohol, and another containing the lipins extracted by toluene. From each of these dishes was made an ether extraction, and the ether used in making the extraction from one dish was mixed with that used in the other dish, then passed through a Berkefeld filter, evaporated to dryness, and stored in a sterile bottle. A hot chloroform extraction and then a hot absolute alcohol extraction were made from the lipins remaining in the two original dishes, the same methods being employed as in the first extraction with ether. All of the lipins went into solution in one solvent or the other, no residue remaining in the dishes. By this method a far larger percentage of lipins was extracted from the bacilli than by Morse's method.

The ether-extracted lipin fraction closely resembled that obtained with ether by the Morse method, but was somewhat darker red in color, with an odor suggesting chocolate. The chloroform-extracted lipin resembled crushed pine rosin, but was rather softer and its fracture was not so glassy. Its odor reminded one of honey. The alcohol-extracted lipin also had a honey-like odor, and in general resembled that extracted by alcohol with the Morse method.

Even after the vigorous measures employed in the Long method of extraction, some lipin yet remained in the bacilli. Twenty-five grams of the whitish, friable cake constituting the residue were put into a two-litre flask, a litre of chloroform was added, and the flask boiled an hour under the reflux condenser. The chloroform was poured off, a litre of toluene was added, and the contents refluxed another hour. The chloroform extracted very little lipin, and the toluene practically none. The bacilli were then assumed to be almost fat-free although many are still acid-fast, but Long (6) has recently shown that even such thoroughly extracted bacilli will still yield a considerable amount of lipoidal material after disintegration with acids.

Grass Bacilli: These were grown on glycerine broth, pH 7, in 500 cc. flasks at 37°C. At the end of about three weeks, when there was a heavy growth, the flask of broth with bacilli was poured into a 1000 cc. graduate and allowed to stand until the bacilli had settled, some sinking to the bottom and others floating on top of the broth. The intermediate broth was pipetted off and the bacilli were left with a small amount of broth; these were washed several times with distilled water to free them from broth and then with 95 per cent alcohol to free them from water. We obtained about 50 cc. of wet grass bacilli by this method, and to this amount we added two litres of cold ether and allowed this to stand over night in the ice-box, then filtered, evaporated and bottled this extract. To the residue we added two litres of alcohol and refluxed for three hours, then filtered, distilled, evaporated and bottled as before. The residue was then boiled under a reflux condenser with two litres of chloroform for three hours, filtered, evaporated and bottled in the same manner as the ether extract. All of the grass bacillus lipins were of a waxy consistency and had a light canary-yellow color.

The residue of grass bacilli was then refluxed three hours with absolute alcohol and again with toluene; but no lipin was removed; so the bacilli were considered fat-free and were dried, bottled and labelled as such.

Colon Bacilli: These bacilli were grown in the same manner as the grass bacilli, but it was necessary to centrifugate the broth with the growth for at least one-half hour at high speed to obtain the bacilli. The broth above the bacilli, which were thus thrown down, was pipetted off and the bacilli were washed in water and alcohol as the grass bacilli were. These bacilli were then extracted with cold ether, hot alcohol and chloroform, but the lipin obtained in each extraction was so small in amount that we combined all the extracted lipins and placed in the same bottle which was labelled *Colon Bacillus Lipins.* This was dark greenish-black in color and formed a rather solid mass with very little odor. The residue of bacilli was treated with absolute alcohol and toluene as with the grass bacilli extraction, and was then bottled, sealed and labelled *Fat-free Colon Bacilli.*

Liver: Two pounds of beef liver were ground in a meat chopper and allowed to stand over night in three litres of 95 per cent alcohol; this was filtered the next day through filter paper and then through a Berkefeld filter, and then placed in a clean bottle. To the residue three more litres of 95 per cent alcohol were added, and this was allowed to stand over night, to be filtered the next day as before. The two filtrates were

mixed and all but about 50 cc. of the alcohol distilled off; this was then evaporated over a water-bath, leaving a dark brownish, greasy mass of lipins, which gave off an indescribably nauseating odor with a suggestion of caramel. This was placed in a clean bottle, sealed and labelled *Cold Alcohol Extract of Liver*. To the residue from the above filtration three litres of cold ether were added and this was allowed to stand in the ice-box over night. This was then filtered and evaporated as above, leaving a golden yellow, unctuous mass which gave off a nauseating, acrid odor. This was bottled, sealed and labelled *Cold Ether Extract of Liver*. To the remaining residue was then added three litres of 95 per cent alcohol and this was boiled for three hours under a reflux condenser, after which it was filtered hot, and the alcohol distilled off and evaporated as the cold alcohol extract had been, leaving a material of golden brown color, soft and granular. This was bottled, sealed and labelled *Hot Alcohol Extract of Liver*. The residue was then refluxed with three litres of chloroform for three hours, after which it was filtered and evaporated as the other solvents had been; but the lipins taken out by this solvent were scanty and of a brownish color tinged with green. This was bottled, sealed and labelled *Chloroform Extract of Liver*. The fat-free residue was then discarded.

METHOD OF INJECTION

One gram of the desired lipin mixture, or fat-free bacilli, was carefully weighed and ground in a mortar with 3 to 5 cc. of 1 per cent sodium stearate solution, to which distilled water was added slowly to 100 cc., until all the material was emulsified. All emulsions thus contained 1 per cent of their respective lipins or fat-free bacilli. These emulsions were preserved in clean bottles, which were plugged with cotton and placed in the autoclave for twenty minutes at fifteen pounds pressure, then removed and the cotton plugs replaced by sterile corks.

Guinea pigs were used in all our experiments, and two injections of 1 cc. were made on each, one subcutaneous injection in the right groin and one intrapulmonary injection between the fourth and fifth right ribs. The animals were all shaved over the area where injection was to be made and washed with alcohol to avoid infection. At least three animals were injected with every emulsion, and observed every day for two weeks. Then they were killed by chloroform or illuminating gas and autopsied.

RESULTS OF INJECTION OF TUBERCLE BACILLUS LIPINS

1. Injection of alcohol-extracted lipins: The lipins extracted with alcohol by both methods produced, subcutaneously, at the site of inoculation, a reddened swelling, followed by subsidence of the swelling and the formation of a small hard nodule, which persisted in only two cases.

Histologically, lipins extracted by both methods produced some endothelial and lymphoid hyperplasia in the regional lymph nodes, and in the subcutaneous tissues a fibroblastic reaction of the epithelioid type. Giant cells, both those with the nuclei arranged in the form of a horseshoe and those with the nuclei clumped toward the centre, were present in the epithelioid tissue produced by lipins of both methods, but were somewhat more numerous in the lesions produced by the Morse-method lipins. Except in one case, in which consolidation of the middle lobe of the right lung occurred, no changes beyond atelectasis were observed in the lungs.

2. Injection of ether-extracted lipins: Subcutaneous injection of lipins extracted by the Long method produced a local swelling and reddening, followed by a small nodule, which regressed to a size barely palpable at the time of autopsy. The inguinal lymph nodes were enlarged. Intrapulmonary injection of this material produced no definite gross lesions.

Lipins extracted by the Morse method produced, subcutaneously, a more severe reaction. The nodules following the primary swelling were much larger than those produced by the Long-method lipins, and one showed a centre of caseoid material. The regional lymph nodes were considerably enlarged. Gross lesions were produced in the lung also, definite consolidations, hard and showing grayish islands. The mediastinal lymph nodes were greatly enlarged, in one case some of them reaching a diameter of 1 cm.

Histological study of tissues taken from guinea pigs injected with Long-method lipins showed no deviation from the normal in the subcutaneous tissue, though the inguinal lymph nodes were somewhat hyperplastic. In the lung, in addition to atelectasis, there was a fibrosis and edema around the larger vessels and bronchi. A great increase in the lymphocytic elements was apparent, in some cases the cells having eroded through the bronchial wall, destroying the continuity of the muscle and cartilage layers, and raising the mucosa adjacent to the break by reason of the large numbers of cells which had wandered in. In one case, in which the material failed to enter the lung, the pleura was

markedly fibrotic and thickened. Nowhere was there any caseation, fibroblastic reaction of epithelioid type, tubercles, or giant cells.

Microscopically, as grossly, the lesions induced by the Morse-method lipins were more severe than those induced by those of the Long method. In the subcutaneous tissue there was a marked fibroblastic reaction of the epithelioid type, the epithelioid tissue containing a few giant cells of both of the types previously mentioned. The substance resembling caseation-necrosis to the naked eye was seen to be merely pus, contained in a large fibrous-walled abscess. Other smaller abscesses, not noted in the gross examination, were also seen in other sections. Some of these were walled off by a fibrous tissue not of epithelioid nature, while some were surrounded by epithelioid tissue containing giant cells. Some sections contained a granulation tissue of definite epithelioid type. The muscle fibres subjacent to the lesions were swollen, vacuolated and hyalinized, and widely separated by the epithelioid tissue which had infiltrated between them.

This same type of tissue was also very abundant in some of the lymph nodes, the normal architecture being obliterated by it. A few giant cells were present. The capsules were greatly thickened.

The lungs injected with the Morse-method lipins showed a beautiful epithelioid type of reaction, in some sections its distribution being general throughout, in others not so widespread. The smaller bronchi and some of the larger ones were full of desquamated epithelial cells and fibrin. The same proliferation of the lymphoid elements produced by the Long-method lipins was observed, and the same erosion through the bronchial walls. The blood vessel intima was somewhat proliferated. On the pleura were both the simple fibroblastic reaction, epithelioid in type, and granulation tissue of similar type. A few giant cells of both types were present, but there was neither caseation nor tubercles.

3. Injection of chloroform-extracted lipins: Subcutaneous lesions produced by lipins extracted by both methods were practically alike. After a primary edematous swelling, a hard nodule appeared early and attained considerable size, in one case being nearly 2 cm. in diameter at autopsy. These nodules on section were found to contain a caseoid mass, which was absent in only one case. The inguinal lymph nodes and lymphatics were enlarged, and in some cases there was induration of the surrounding fat tissue.

The gross lesions produced by intrapulmonary injection of lipins, extracted by both methods, also closely resembled each other. In all

cases consolidations were produced, and there were extremely dense and extensive fibrous pleural adhesions.

Histological study showed that hyperplasia was produced in the regional lymph nodes by injection of lipins of both methods. In addition, the Morse-method lipins produced hyperemia in some nodes. There was also a perfectly typical fibroblastic reaction, epithelioid in type, in some of the lymph nodes of guinea pigs receiving the Long-method lipins. An occasional giant cell was present here.

In the subcutaneous tissues, the lipins of both methods produced a purulent inflammation, sometimes phlegmonous in character, sometimes in the form of abscesses definitely walled off by either typical epithelioid tissue or granulation tissue of similar character, or both. Very few giant cells were present, but no caseation.

The chloroform-extract lipins produced on the pleura both a granulation tissue of epithelioid type and the usual fibroblastic reaction of similar type. In the lung itself, where the alveoli were not obscured by the proliferation of epithelioid cells or atelectasis, the alveolar walls were thickened, the epithelium desquamated, and the alveoli jammed with these cells and exudative materials. In some sections small areas closely resembling tubercles were seen, consisting of a central mass of cellular debris, staining with both hematoxylin and eosin, then an area containing only a few radially arranged epithelioid cells, the whole surrounded by the definite tissue of epithelioid type, which contained a few giant cells, although none were observed in the centres of these tubercle-like areas. Other areas present in the epithelioid tissue suggested small abscesses. There were also hemorrhagic areas, sometimes quite large, in the epithelioid tissue. The erosion through the bronchial wall, with lifting up of the mucosa, seen in previous cases, and accompanied by or due to a great proliferation of the lymphocytic elements of the lung, was also observed. A slight bronchitis with desquamation and fibrinous exudation was present. No areas suggesting caseation were seen in any specimens. The effects differed little with the chloroform extracts of the two methods.

4. Injection of defatted bacilli: In all cases a hard nodule succeeded the primary inflammatory reaction resulting from the injection. In only one case was caseoid pus visible in the gross specimen. In all animals the regional lymph nodes were somewhat enlarged, and in one the axillary nodes also. The lungs showed in two cases hard, grayish, nodular consolidations with grayish islands on section, but in two others no gross consolidations were evident.

Histologically, the regional lymph nodes were hyperplastic. Subcutaneous tissue taken from the inguinal region showed small areas of suppuration not noted macroscopically. The nodule described as containing caseoid pus was simply a larger abscess. Sometimes necrotic and hyaline muscle fibres appeared in the centres of these suppurating areas, surrounded on all sides by dead and disintegrated polymorphonuclear leucocytes, these in turn being surrounded by the fibroblastic reaction of epithelioid nature, in which case the area of polynuclear leucocytes merged gradually with the epithelioid tissue, or was surrounded by granulation tissue of similar type or by a tissue which was hard to distinguish from common granulation tissue. More giant cells than seen before in any section were present. The tissue was literally studded with them. Numerous epithelioid tubercles were scattered throughout, but none contained any giant cells.

On the pleura there was a fibroblastic reaction of epithelioid type, granulation tissue of the same nature and in some sections a dense hyaline scarring. In the lung itself both epithelioid granulation tissue and the simple epithelioid tissue were observed, the latter showing several epithelioid tubercles and numerous giant cells, and in some cases containing large areas of hemorrhage. No caseation was observed in any case.

INJECTION WITH LIPINS FROM GRASS BACILLI AND COLON BACILLI

The effects produced with these preparations were very similar to those obtained by injection of corresponding extracts of tubercle bacilli, and therefore need not be discussed in detail. In general, they produced a fibroblastic reaction of epithelioid type in the lungs and the subcutaneous tissues; but the ether and the chloroform extracts of the tubercle bacilli produced a similar reaction in the lymph nodes nearest the site of injection in some cases, which none of the nonpathogenic extracts or defatted bacilli were able to do.

In the lesions following the injection of nonpathogenic extracts and defatted bacilli, as well as in those following similar injections of tubercle bacillus extracts and defatted bacilli, there were numerous giant cells of the typical and foreign body type, these being a great deal more numerous in the tissues injected with the defatted tubercle and grass bacilli. These two emulsions, namely, the defatted tubercle and grass bacilli, produced very similar tissue reactions; but the defatted colon bacilli had a marked tendency to cause a scab formation and a superficial ulceration

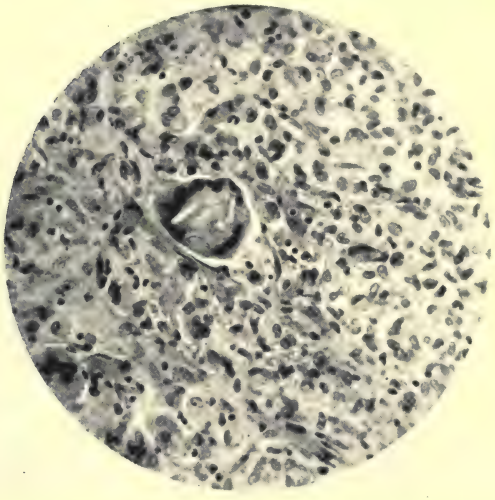

FIG. 1. LESION PRODUCED BY INJECTION INTO THE SUBCUTANEOUS TISSUES OF THE LIPINS EXTRACTED FROM COLON BACILLI. × 200

Note the similarity of the cellular reaction, including giant cell formation, to the tissue changes of tuberculosis. Similar lesions were produced by the lipins from tubercle bacilli and from grass bacilli, but not with the lipins from beef liver.

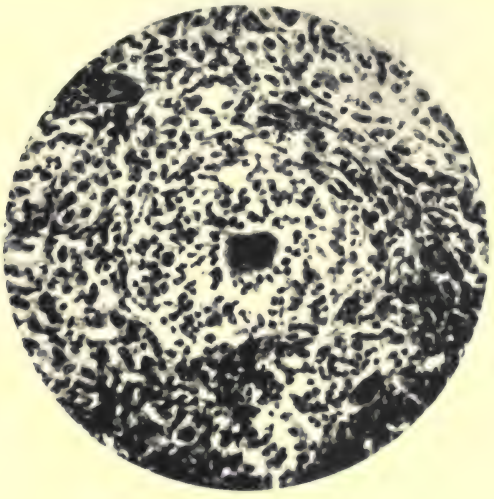

FIG. 2. LESIONS RESEMBLING ACTINOMYCOSIS PRODUCED BY SUBCUTANEOUS INJECTION OF DEFATTED GRASS BACILLI. × 200

in the groin of the animals injected, and microscopically these lesions did not show any giant cells and the fibroblastic reaction was not so marked as in the lesions produced by the defatted tubercle or grass bacilli, although there were many areas of abscess formation as in most of the lesions produced by the extracts of other bacilli.

The lipins of the colon bacillus produced quite the same effects as the lipins of the acid-fast bacilli, but less in degree and with a smaller number of giant cells (fig. 1).

The formation of purulent areas, filled with disintegrating polymorphonuclear cells and necrotic tissue, surrounded by a fibroblastic capsule and sometimes giving the appearance of actinomycotic lesions (fig. 2), was a rather constant finding in the lungs and especially in the subcutaneous tissues, following the injections of lipins from both tubercle and nonpathogenic bacilli and also injections of defatted bacilli. We cannot corroborate the statement of Auclair (7) that ether extracts of different bacteria produce the lesions that are characteristic of infection by each microörganism.

The chloroform extract of the tubercle bacilli and also of the nonpathogenic bacteria produced the most marked lesions of all the extracts, but only in the tissues injected with the chloroform extract of tubercle bacilli were definite epithelioid tubercles commonly produced. In few cases were these typical tubercles produced by the injection of nonpathogenic extracts or nonpathogenic defatted bacilli.

EFFECTS PRODUCED BY INJECTION OF FATTY MATERIALS FROM BEEF LIVER

These were much less than the effects produced by corresponding extracts from any of the three sorts of bacilli, as seen by the following description of the lesions observed in the injected animals.

1. Cold alcohol extract: Produced no lesions in lymph nodes, and a very small amount of fibrous reaction in the subcutaneous tissues near the site of injection in the right groin. The lungs showed a light desquamation of the bronchial epithelium and some atelectasis.

2. Hot alcohol extracts: Did not affect the lymph nodes, and the subcutaneous tissues at the site of injection in the groin showed only a slight fibrous reaction. The lungs showed an increase in small lymphocytes, some of which encroached upon the bronchial walls and broke through them in places. The bronchi showed a desquamating and exudative bronchitis.

3. Ether extract: Produced no visible lesions other than atelectasis of the lungs.

4. Chloroform extract: Caused some hyperplasia of the lymph nodes. Sections from the subcutaneous tissues and muscles in the region of the injection in the groin showed a proliferation of fibroblasts, giving rise to an indistinct fibrous capsule which surrounded what we took to be a chemical abscess, full of disintegrating polymorphonuclear cells. In some sections from this region the fat tissues were somewhat infiltrated by a fibroblastic tissue of epithelioid type, in which there was an occasional giant cell of foreign body type. Sections from the lungs showed a fibrous thickening of the pleura and many eosinophiles. There was a fibrosis about some of the bronchi and a slight amount of fibrous tissue of epithelioid type in parts of the lung tissue. There were also some areas of atelectasis, but none of the extensive epithelioid and fibroblastic proliferation produced by the bacillary lipins.

In conclusion, we can say that in no case did we produce true microscopic caseation, as Gaehlinger and Tilmant claim to have done with liver lipoids.

CONTROLS

In order to make sure that the results obtained were due to the fatty materials under consideration and not to the solvents injected with them, the following experiments were performed.

1: Injected a guinea pig in the lung and groin with 1 cc. of 0.25 per cent sodium stearate emulsion. This was much stronger than the concentration of sodium stearate used in emulsifying the lipins, in which from 3 to 5 cc. of a 1 per cent emulsion to 100 cc. of distilled water was employed, making the maximum concentration 0.05 per cent. The tissues at the site of injection were normal in every respect at the end of two weeks.

2: Injected in lung and groin with 1 cc. of 0.25 per cent sodium stearate emulsion, containing 0.5 per cent of chloroform. The lungs were normal. The skin over the area of injection in the groin was slightly thickened and edematous, but the reaction was a simple fibrosis and not even suggestive of the epithelioid type. No giant cells were present. The chloroform was injected because of the fact that the extensive lesions produced in the case of the chloroform lipins might have been accentuated by the small amount of chloroform which might possibly be present in the lipins extracted.

3: Injected in lung and groin with pure olive oil containing 0.5 per cent chloroform. This mixture was injected because of the possibility that the chloroform might produce more extensive lesions if injected in a menstruum which would delay absorption. There were no lesions, however.

4: Injected in lung and groin with 1 cc. of olive oil alone. In this case a slight amount of fibrosis in the subcutaneous tissue was produced. The lung was normal.

DISCUSSION

Morse and Stott found that the ether-soluble lipins of the tubercle bacillus were inert unless injected in amounts sufficient to cause pulmonary embolism. They, however, used white rats and rabbits as experimental animals, and it is possible that these animals are not susceptible to the action of the ether-soluble lipins, for our ether-soluble preparations were decidedly active in the guinea pig. A possible difference in the composition of the ether-soluble lipins, as extracted by Morse and as extracted by ourselves with his method, might account in some measure, though not entirely, for the differences in the results following injection of this lipin. Morse described his ether extract as a dark, viscous oil; ours was a dark red, waxy solid. The bacilli were allowed to stand in the cold ether for twenty-four hours by Morse, whereas we waited only twelve hours before pouring off the ether. This difference in the appearance of the extract may then be due to a more complete evaporation on our part, or to an actual difference in composition of the two extracts, it being conceivable that there may have been present in the bacilli some substance slowly soluble in ether, which by its presence in the final extract would change its physical properties and to some extent its pathogenic powers.

It is to be noted that, in our own experiments, using one species of animal throughout, and with the conditions for one animal being identical for all, our results varied; for instance, in only one case did any gross lesions result from intrapulmonary injection of alcohol-soluble lipins extracted by the Long method. In all experiments one must, of course, take into account the factor of individual resistance.

It was observed that lesions produced by the injection of lipins extracted by one method sometimes differed from the lesions produced by injection of the lipins extracted with the same solvent by means of the other method. The lesions following injection of ether-soluble tubercle

bacillus lipins, for example, extracted by the Morse method, were more severe than and differed from the lesions produced by the ether-soluble lipins extracted by the Long method. Further, it was noted that, in general, the epithelioid type of tissue seen in lesions induced by the Morse-method lipins was more vascular than that seen in the lesions caused by injection of Long-method lipins. There was also more hemorrhage associated with the lesions following injection of Morse-method lipins than was observed in lesions following injections of lipins extracted by the Long method.

It is hardly probable that the extracted lipins existed in the same chemical structure in the bacilli before extraction. Surely the heat and the succession of solvents would cause some molecular rearrangement, some change in melting point, or some change in solubility, so that the final product would differ greatly from the original material as contained in the bacillus. And, of course, different methods of extraction would cause different changes; the alcohol extract of tubercle bacilli, for instance, might contain different lipins or groups of lipins when the Morse method was used than they would contain when some other method was used. We must fall back upon some such explanation as this for the variations in the lesions produced. If the chemical structure of the tubercle bacillus were definitely known, and if it were possible to extract each separate constituent or group of constituents, and inject each separately, definite statements as to the effect of each substance injected could of course be made. But at present each observer uses a different method of extraction, which gives different products, and so results must vary. Until we know what we are injecting, statements as to the effects of injection of "alcohol-soluble lipins" or "ether-soluble lipins" of the tubercle bacillus must be regarded as being merely a shot in the dark as far as regards specificity of the materials injected.

Notwithstanding this lack of exact knowledge regarding the substances injected, there was a remarkable similarity in the tissue response to injections of lipins from the bacilli and the defatted bacilli themselves. With the exception of the presence of more giant cells and epithelioid tubercles in the lesions induced by injection of defatted bacilli, the lesions were alike. Indeed, one could not say with certainty from the microscopic picture alone, whether a given section was taken from an animal injected with tubercle bacillus lipins, defatted nonpathogenic bacilli, or living tubercle bacilli. Of course, the living tubercle bacilli

would in time produce caseation, which was absent in the lesions produced by the defatted bacilli and the lipins removed therefrom, but in the earlier stage the similarity of the lesions produced by these three agents is extremely impressive. One often sees uncaseated tuberculous lesions in the tissues of guinea pigs that do not differ at all from those produced by these lipins.

How can one explain this marked sameness in the tissue response to the injection of tubercle bacillus lipins, lipins from other bacilli, the defatted bacilli, and the intact (living or dead) bacilli? Shall one assume the presence of a toxic material which is present in all the extracts, as well as in the bacilli after the extraction? Or shall one assume that the fibroblastic reaction of epithelioid type, the tubercles, and the giant cells are merely the normal response of the body to an irritating foreign substance?

Since we obtained a tissue response with lipins from grass bacilli and colon bacilli, as well as with the defatted bacilli, exactly identical in all important respects to that obtained with tubercle bacillus lipins and fat-free tubercle bacilli, and since it is well known that subcutaneous injections of paraffin and many other substances frequently invoke a similar tissue response, it would seem that the latter assumption is the more likely explanation.

As far as we know, Auclair is the only investigator who has reported the production of caseation by means of injection of lipins of the tubercle bacillus. Since there exists in the serum of rabbits such quantities of antienzyme, antagonistic to the proteolytic enzyme liberated from the polynuclear leucocytes, that pus in these animals is hard and firm, suggesting in this respect caseation (8), and since Auclair used rabbits as well as guinea pigs in which this condition exists to a less degree, we must conclude that Auclair's "caseation" was simply solid pus. Fur ther, his description of the microscopic appearance of his caseation is far from convincing.

It would seem, then, that the body's response to the presence of the tubercle bacillus is not a specific defense built up by the invaded body, but merely a type defense,—a reaction invoked by many and varied substances, and in no way peculiar to tuberculous lesions. Apparently, the caseation seen in actual infection with the tubercle bacillus is due to the diffusion of some toxic product of bacillary metabolism, and is seen only when the bacilli are alive and the inflammation progressing (9).

SUMMARY

1. Tubercle bacillus lipins and the defatted bacilli possess the power of exciting a local fibroblastic reaction of epithelioid type, with tubercles, giant cells, and abscesses, when injected into the lungs and subcutaneous tissues.

2. Hyperplasia of the regional lymph nodes was constant. Fibroblastic proliferation of epithelioid type was produced in the regional lymph nodes only by the ether and chloroform-soluble lipins.

3. With the ether-soluble lipins extracted from tubercle bacilli by two methods, and the chloroform-soluble lipins extracted by the Long method, sufficient proliferation of the lymphocytic elements of the lung was produced to erode the bronchial walls.

4. Definite epithelioid tubercles were produced only with the chloroform-soluble lipins and with the defatted bacilli. Far more definite tubercles were present in lesions induced with the defatted bacilli than in lesions induced with the chloroform-soluble lipins.

5. Giant cells were present in the lesions produced by injections of all the bacterial lipins tested, but were far more numerous in lesions produced by means of the defatted acid-fast bacilli.

6. In general, the lipins extracted by the Morse method produced a more vascular epithelioid tissue, with more associated hemorrhage, than was produced with lipins obtained by means of the Long method.

7. Lipins extracted from grass bacilli and colon bacilli by similar methods produce effects entirely similar to those produced by the tubercle bacillus, except that with the latter there was more involvement of the regional lymph nodes. Evidently, then, the characteristic histological features of tubercle cannot be considered to be specific effects of the lipins of the tubercle bacillus, but merely a foreign body reaction, perhaps slightly modified by the water-soluble products of tubercle bacilli.

8. Defatted tubercle bacilli and grass bacilli may produce typical epithelioid cell lesions with many giant cells. Lesions identical with those of actinomycosis may also result. Defatted colon bacilli produce similar lesions but with no giant cells.

9. No areas of true caseation were observed in any section. Probably the supposed production of caseation with lipins from tubercle bacilli and beef liver reported by others depends on the solid character of the pus produced in rabbits and guinea pigs, which macroscopically closely resembles caseous material.

REFERENCES

(1) Auclair: Étude expérimentale sur les poisons du bacille tuberculeux humain, Thesis, Paris, 1897, no. 321.
(2) Auclair: Rev. d. l. Tuberc., 1898, 97.
(3) Auclair: Arch. Méd. Exp., 1899, xi, 363; 1900, xii, 189.
(4) Morse and Stott: Jour. Lab. & Clin. Med., 1916, ii, 159.
(5) Gaehlinger and Tilmant: Compt. Rend. d. Soc. Biol., 1911, lxxi, 345.
(6) Long: Amer. Rev. Tuberc., 1922, vi, 642.
(7) Auclair: Arch. Méd. Exp., 1903, xv, 725.
(8) Opie: Arch. Int. Med., 1910, v, 541.
(9) Dürck and Obendorfer: Ergebnisse der Path., 1899, vi, 288.

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STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS

XXVI. FAT-SPLITTING FERMENTS IN LYMPHOCYTES

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The prominence of the lymphocytes in the body's reaction to infection by the tubercle bacillus has naturally led to inquiries as to the part they may play in this defense. In 1909 Bergel (1) reported experiments which were interpreted by him as establishing the presence in lymphocytes of active fat-splitting ferments, to which he attributed their supposed capacity to attack the tubercle bacillus.

Tuberculous exudates and spleen and lymph node juice were placed on plates and allowed to stand in an incubator at 52°C. for twenty-four hours. The plates were made from stearin, palmatin, and white and yellow wax, the latter with a melting point of 63° to 64°C. It was not specifically stated whether or not the yellow and white wax was beeswax, but presumably it was. The plates showed no digestion or depression in the stearin or palmatin, and various lymphocytic materials simply dried up. On the yellow wax plates, especially, characteristic depressions with crater-like hollows appeared and these were surrounded by a well defined ridge or wall, so that the wax had the appearance of having been digested or eaten out by the lymphocytes.

Control tests were made, using, in place of the lymph materials, physiological salt solution, tuberculin, fibrin and trypsin, all of which dried up and gave no depression in the wax.

The production of these hollows with the raised margins was interpreted as indicating that the lymphocytes had digested the wax, and, on the basis of these observations and this conclusion, the suggestion was made that the importance of lymphocytes in tuberculosis lies in their capacity to attack the waxy constituents which form so large a part of the substance of the bacillus. This hypothesis has been conspicuous in the tuberculosis literature since Bergel's paper, although it has not been submitted to very extensive reinvestigation.

Caro (2) performed some experiments with monobutyrin and tributyrin, which he found to be split by lymphocytes, but apparently he did not repeat Bergel's identical experiments. From his own observations and the reports in the literature he drew the conclusion that it has not yet been positively shown that lymphocytes contain fat-splitting ferments.

Nees (3) carried out a large number of experiments similar to Bergel's. Beeswax alone was used in part of the experiments and mixtures of beeswax and various other substances, such as paraffin, hog fat, etc., together with an indicator, methyl red, were used in the last part of the work. The cellular exudates from a wide variety of disease conditions were placed on the different kinds of plates. The results obtained by Nees were not constant. Sometimes some plates in the same series with the same kind of cell material gave results indicating a splitting of the fat, while other plates in the same series showed no change. There was no explanation offered for these discrepancies or differences. Nees's conclusions were (1) lymphocytes as well as leucocytes split fat, (2) the ability to split fat is a general cell property, and (3) caution must be used in judging results, especially in applying them to tuberculosis as Bergel had done. Since then Bergel has published other articles (4), which he believes also support the hypothesis that lymphocytes are important in tuberculosis because of their ability to attack fats and waxes. Recently Aschoff and Kamiya (7) have taken exception to Bergel's claims that he has shown that lymphocytes possess any characteristically high capacity for the disintegration of lipoids, but they did not reinvestigate his experiments with the wax plates.

As we have on hand in our laboratory considerable quantities of different lipins extracted from tubercle bacilli, it was suggested that it would be desirable to determine whether the enzymes of lymphocytes which split other fats and waxes are capable of attacking the lipins of the tubercle bacillus. This would serve to throw light on the reputed significance of lymphocytes in tuberculosis as destroyers of tubercle bacilli through their ability to attack the latter's waxy material. I therefore undertook, first, to repeat Bergel's experiments, in order to acquire familiarity with his technique and results, before applying the same technique to the lipins obtained from tubercle bacilli.

EXPERIMENTAL METHODS

In my preliminary experiments the tissues containing lymphocytes were macerated in fine sand and about 10 cc. of tap water were added

for each 2 gm. of tissue. Five cubic centimetres of the resulting emulsion was then boiled on a water bath for one-half hour in order to destroy the enzymes. This portion was used for control experiments. To each 5 cc. portion was added 0.5 cc. of toluene to prevent putrefaction, and small drops of each emulsion were then placed on plates of the different materials to be tested.

In the second set of experiments the different lymphoid tissues to be tested were placed directly on the wax plates without making an emulsion with water or adding toluene.

Finally, the lymph nodes were scraped with a knife, in order to obtain cellular material, and this was placed on the wax plates, for it was thought that by so doing the "sinking in" of the solid tissue could be eliminated and the exact effect of the lymphocytes could be observed.

The plates were glass containers, about 3 cm. in diameter and 1 cm. deep, with straight sides and flat bottoms. The different fats and waxes were melted and poured into these plates and allowed to cool to room temperature before the tested materials were placed on the smooth top surface of the wax. In some of the experiments methyl red was added directly to the melted fat or wax before pouring into the plates, according to the method used by Nees. After placing the material evenly on the plates, they were carefully covered with glass lids and the edges sealed with melted beeswax. The plates were then placed in a porcelain dish containing a little water and the dish in turn was covered with a lid and its edges carefully sealed with melted beeswax. It was found necessary to do this to prevent the lymph material from drying, especially when incubated at 52°C.

RESULTS OF EXPERIMENTS

1: In the first set of experiments the materials used were from guinea pigs. They comprised (1) normal lymph nodes, (2) normal spleen, (3) caseous, tuberculous spleen, and (4) caseous, tuberculous peritoneal lymph nodes. The material was made into an emulsion and toluene was added. Controls were run with boiled material. Beeswax and lanolin were used, and three plates made in each case, two for the normal tissue and one for the boiled, and each incubated at 37°C. for twenty-four hours.

No visible change resulted in any of the beeswax plates. The emulsion sank into the lanolin in every case, whether of boiled or fresh tissue, but there was no visible digestion.

2: A second set of experiments was carried out as above, with the exception that methyl red was added to the plates prepared from lanolin, stearin and beeswax. The tissue used here was from normal guinea pig pancreas and lymph nodes.

The plates showed no digestion in any case and no color reaction on any, except on the stearin plates with the unboiled pancreas emulsion, which showed a bluish ring about the drops of emulsion.

3: In place of the emulsified tissue material, the tissue was placed in small pieces directly on beeswax, lanolin, and half lanolin, half beeswax plates, to which methyl red had been added, and these plates were then incubated forty-eight hours at 52°C.

The plates showed no color change, but there was a slight depression in all plates, with the boiled as well as with the fresh material.

4: Plates were also made from the wax or fat from tubercle bacilli prepared according to Long's method (5), chloroform soluble fraction, and also from beeswax.

The material used here was normal guinea pig (1) pancreas, (2) spleen, (3) lymph nodes, and also tuberculous human lymph nodes. The plates in each case were kept at 37°C. for twenty-four hours and a second set at 52°C. for forty-eight hours. They showed a slight depression in the beeswax and considerable depression in the tubercle wax, even with the boiled lymph material. The depressions were deeper in the plates incubated at 52°C.

5: Plates were made as in *3* and *4*, but the cellular material was secured by scraping the lymph nodes with a knife. Inert substances, such as beeswax and small pieces of wood, salt solution and water, were used for controls, and the plates were incubated forty-eight hours at 52°C. There was a slight depression with each material, whether fresh cells or inert substances.

DISCUSSION OF RESULTS

Early in the work it was noticed, especially with the lanolin and later with the wax of the tubercle bacilli, that the lymphatic material had sunk down into the plates, and at first it was thought that this was due to digestion of the fat by the enzymes present in the lymphocytes. But when boiled tissues, and later even inert material like beeswax, water and salt solution, also sank in when incubated, it became evident that there was no digestion at all, but rather the depressions in the wax

were really due to the "sinking in" of the materials used in the incubator, especially since this was more noticeable at 52°C. No difference whatever could be discerned in respect to the character of the depression produced by fresh tissues and inert controls. Indeed, when some of the plates of beeswax and tubercle wax were compared with the description of results and a cut given by Bergel (1), it is easy to see how this "sinking in" of lymph material could be mistaken for a digestion. The raised margin or rim about the depression in the wax is probably compensatory to the sinking in of the surface, *and is of itself evidence that the depression is simply mechanical rather than the result of digestion.*

The results obtained by using the pancreas as a source of lipase are especially important. It was found that the only evidence of fat splitting or fat digestion, even with this lipase-rich organ, was with stearin. However, there was no characteristic "walled about depression" here, such as Bergel describes, only a slight change to acid indicating a breaking down of the fat molecule. With stearin, lymph nodes gave no color reaction. Indeed, in no other experiments was there seen any change in the indicator which would indicate digestion.

That negative results were obtained is probably due to the fact that the wax molecule, in the case of each of the waxes used, was too stable to be attacked by lipase even if this enzyme were abundant in the lymphocytes. This will be made evident from a consideration of the chemistry and hydrolysis of beeswax.

According to Lewkowitsch (6), in order to hydrolyze beeswax it requires one hour of boiling with strong alcoholic potash made with at least 96 per cent alcohol. The difficulty with hydrolyzing the solid waxes is probably due to the fact that the resulting sodium soaps are less readily soluble than those obtained from hydrolyzed fats, and hydrolysis does not occur in steps or stages as in the case of simple fats. In our experiments even fresh pancreas produced no evident digestion of beeswax.

From these considerations it is seen that beeswax offers a considerable resistance to measures that are commonly efficacious in hydrolyzing ordinary fats and, therefore, it would appear unlikely that beeswax could be digested by ordinary tissue enzymes.

Beeswax has many properties similar to the fatty substances which form a considerable part of the bodies of tubercle bacilli. These fatty substances not only exhibit a very low iodine value but have a strong resistance to measures that commonly saponify ordinary fats.

CONCLUSIONS

1. There was no demonstrable digestion or fat splitting of the wax of tubercle bacilli, beeswax or lanolin by material containing lymphocytes when tested by the plate method of Bergel. This is thought to be due to the fact that the waxes are so stable that they cannot be hydrolyzed or digested by ordinary tissue enzymes. No digestion was observed even with fresh pancreas, which is known to contain strong lipase.

2. The depressions that were observed in the wax plates were apparently due entirely to sinking in of the tested material, while the wax was softened in the incubator, and not due to a digestion of the wax, since depressions similar in character were produced by boiled tissues and cells, as well as by entirely inert substances.

3. There was no change in reaction (as shown by the methyl red indicator) produced by any of the materials containing lymphocytes on tubercle bacillus wax, stearin, beeswax or lanolin, either in twenty-four hours at 37°C. or in forty-eight hours at 52°C., as would be expected if there was any digestion or hydrolysis of the wax.

REFERENCES

(1) Bergel: Fettspaltendes Ferment in den Lymphozyten, Münch. med. Wchnschr., 1909, lvi, 64.

(2) Caro: Fettspaltende Fermente im menschlichen Blutserum, Ztschr. f. klin. Med., 1913, lxxviii, 286.
Zur Frage der Herkunft und Bedeutung von fettspaltenden Fermenten des menschlichen Blutes, Ibid., 1920, lxxxix, 49.

(3) Nees: Über die lipolytische Fähigkeit der weissen Blutkörperchen, Biochem. Ztschr., 1921, cxxiv, 156.

(4) Bergel: Beiträge zur Biologie der Lymphozyten, Ztschr. exp. Path. u. Therap., 1920, xxi, 216; Berl. klin. Wchnschr., 1921, xxxiv, 995.

(5) Ray and Shipman: Amer. Rev. Tuberc., 1923, vii, 88.

(6) Lewkowitsch: Chemical technology of oils, fats and waxes, MacMillan Co., London, 1914, vol. 1, pp. 65–68, and vol. iii, 900–920.

(7) Aschoff and Kamiya: Deutsche med. Wchnschr., 1922, xlviii, 794.

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HEMATOLOGICAL STUDIES ON EXPERIMENTAL TUBERCULOSIS OF THE GUINEA PIG[1]

I. BLOOD MORPHOLOGY, ARNETH COUNTS AND COAGULATION TIME IN NORMAL AND UNTREATED TUBERCULOUS GUINEA PIGS

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. XXVII

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This work was undertaken to determine whether changes in the blood might serve to indicate the effect of methods used in chemotherapeutic experiments on tuberculous animals. The present report consists of the results of examinations made on the blood of normal and untreated tuberculous guinea pigs. We are now making similar examinations of treated tuberculous animals, for diagnostic and prognostic purposes, in testing the value of the drugs under experiment. A report of this work will appear later.

A review of the literature of studies on the blood in human and experimental tuberculosis shows little unanimity as to the blood changes that occur in this disease. Indeed, this was to be expected on account of the complexity of the disease itself and the differences in its manifestations in tuberculous adult patients, who have been studied in most cases, and in the nonsensitized guinea pigs that are used for experimental work.

REVIEW OF THE LITERATURE

On human tuberculosis:

The earlier hematological studies in human tuberculosis have been carefully reviewed by Ullom and Craig (1) and Klebs and Klebs (2). These reviews bring out the complete lack of uniformity in the results of routine clinical blood counts in tuberculosis. Although it is fairly generally agreed that a moderate anemia of the secondary type prevails, in which the decrease

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of hemoglobin is relatively greater than that of the red blood cells, there is no agreement as to whether the latter are increased, normal or decreased in the first and second stages. Even in the third stage, normal or increased counts are reported by some workers, though a progressive decrease of red corpuscles is reported by the majority.

Sokolowski (3) and Applebaum (4) claimed that variations in the red blood count, with frequent increases of erythrocytes above normal, are common, and are due to changes in the blood concentration from profuse sweatings, expectoration and diarrhea. But Grawitz (5) believed the variations were due to the effects of the bacilli which cause a transudation of serum from the tissues into the blood.

Irregular variations were also found in the leucocytes, but it was generally thought that an increase in the neutrophilic polymorphonuclear elements indicated the advent of a mixed infection and that such were found more commonly in the later stages.

Neither do workers agree as to the reaction of the lymphocytes to tuberculosis. Thus, Ewing (6) found an excess in all mononuclear cells in acute and most chronic cases; but Strauss and Rohnstein (7) were impressed with the fact that the mononuclear percentage tended to decrease with the progress of the disease. While the results of the counts at the Phipps Institute (1) seemed to indicate that an actual increase in lymphocytes corresponded to an increase in resistance, Ullum and Craig believed that the point was not fully established.

Reports of the reactions of eosinophiles are also inconstant. Several workers found them normal in the early stages, but absent or decreased in severe cases. Zappert (8) believed them to be decreased in febrile cases, but increased with or before a fall in temperature. Halborn (9) thought that an increase in eosinophiles indicates resistance to the disease. Swan (10) believed that an eosinophilia was prognostically favorable.

More recently there has been less analysis and publication of routine blood counts; but we find Solis-Cohen and Strickler (11) reporting an increase of lymphocytes with improvement in pulmonary tuberculosis and an increase of polymorphonuclear cells as the patient grows worse. Weil (12) found a steady decrease in lymphocytes and eosinophiles as the disease progressed. Yokoyama (13) found the red blood count and hemoglobin normal until the third stage, when they both decreased, but the hemoglobin more rapidly. Lymphocytes decreased as the disease advanced. Eosinophiles were normal until the last stage when they were slightly decreased. McDougal (14) suggested that the rapid variations in leucocytes might be due to chemotactic or anaphylactic influences and that this so called "leucocytic swing" might have diagnostic and prognostic value. Cummings (15) believed that a high percentage of lymphocytes was indicative of an increased resistance.

From these reports the tentative conclusions might be drawn that in clinical tuberculosis there frequently appears a mild chlorotoid anemia, a variable red blood count possibly due to changes in the blood concentration, a leucocytosis in cases of secondary infection, and a lymphocytosis and eosinophilia if a resistance is developed to the tuberculosis.

On experimental tuberculosis:

In recent years there has been an increasing number of studies on the changes of the blood picture in experimental tuberculosis. In 1901 Achard and Loeper (16), working on rabbits and dogs, noted a primary increase in the polymorphonuclear elements, which subsided in a few days and was followed by an increase in eosinophiles and mononuclear elements. A polymorphonuclear leucocytosis might reappear later. Claude and Aly-Zaky (17) found that the eosinophiles appeared in the beginning of the infection and remained high in circumscribed fibrous cases, but disappeared rapidly in acutely advancing cases. Gaertner and Roemer (18) showed that extracts of tubercle bacilli, injected intravenously into dogs, produced a change in the lymph flow, which they believed to be caused by an alteration in the relation between the blood vessels and the tissues in the interchange of lymph. Hussey (19) concluded that guinea pigs, rendered relatively immune to virulent tuberculosis by avirulent bacilli, show an increase in lymphocytes, as they did also in avirulent tuberculosis, and that the greatest activity of the lymphocytes occurred at the period of greatest reaction to the infection. No such lymphocytosis, but an irregular leucocytosis, occurred in virulent tuberculosis. Rogers (20) found a fall in lymphocytes which became quite marked in the later stages. A preliminary sensitization with an avirulent strain of tubercle bacilli, followed by inoculation with virulent bacilli, caused little or no change in the leucocyte picture from that observed in nonsensitized animals. Murphy and Sturm (21) found that tuberculous guinea pigs exposed to dry heat showed an increase in lymphocytes and an increased resistance to tuberculosis. But Corper and Gauss (22) in a similar experiment observed that all animals, whether exposed to dry heat or not, showed a steady decrease in lymphocytes. Taylor and Murphy (23) found that guinea pigs, in which lymph tissue and lymphocyte count had been reduced by X-rays, were more susceptible to tuberculosis. In the last stages of tuberculosis in guinea pigs McJunkin (24) found an increase in endothelial leucocytes which persisted until the animal died.

METHODS AND TECHNIQUE

Our experiments were carried out on healthy male guinea pigs, of uniform weight and inoculated subcutaneously in the inguinal region with pure cultures of human tubercle bacilli. They received no other treat-

ment and were left to the usual laboratory care until death, after which they were carefully autopsied. Normal animals, living under the same conditions but in different cages, were also studied as controls.

Routine blood counts were made at intervals of from one to three weeks, the shorter periods being used in the acute cases and the longer ones in the chronic cases or normal animals. The study covered a period of twenty-one months, from April 1, 1921, to January 1, 1923. Many of the chronic cases and normal animals were examined during this whole period. Over 700 complete blood examinations, besides several special studies discussed later, were made. Forty animals were studied throughout their disease until death. Seven normal animals were examined over the same period, and isolated counts made on a score of other normal animals.

The usual clinical methods were employed. Blood was obtained by pricking the large vessels of the ear. Red and white corpuscles were counted in Türck counting-chambers, using Hayem's solution and 0.3 per cent acetic acid with methyl violet. Hemoglobin was read by the Sahli instrument. Coverslip smears were stained with Wright's stain. All examinations were made in duplicate, and if the duplicate counts did not agree a third examination was made. Leucocytes were classified as lymphocytes, large mononuclears, transitionals, neutrophilic polymorphonuclears, eosinophiles, and basophiles. In making the charts the various leucocytes were charted in their calculated absolute values and not their relative or percentage values.

Animals were classified as (I) *Normal*, or uninoculated controls; (II), *Acute*, which, by definition, were those which received an inoculation of tubercle bacilli that caused them to die of generalized tuberculosis in less than four months; and (III) *Chronic*, those animals that were inoculated with tubercle bacilli and at some time showed enlarged, palpable regional lymph nodes or in which tuberculous lesions were found at autopsy, but which did not die of tuberculosis in less than nine months. These three groups will be discussed separately.

STUDIES ON NORMAL ANIMALS

Seven normal animals were examined during the period of experiment, a total of 130 complete counts being made on them. Isolated counts were made on a score of other normal animals (see nos. 3 and 20, charts 1 and 2, and table 1). The following normal standard was established

for male guinea pigs, of from three months to two years of age and living in our laboratories.

These results approximate closely those obtained by other workers, as seen in the table in Burnett's *Clinical Pathology of the Blood of Animals*. The red blood count is there given as 5,276,000, Burnett (26); 5,859,000, Hayem (27); 5,780,000, Kurloff (28); and 4,240,000, Cohenstein and Zuntz (29). Burnett found a hemoglobin reading of 94.5 per cent. Leucocyte counts were 7,000, Bethe; 11,000, Burnett; 5,600, Hayem; and 12,600, Kurloff.

The differences between normal guinea pig and normal human blood are most noticeable in the higher red blood cell count and hemoglobin percentage and the markedly higher lymphocyte count in the guinea pig. There is also less variability in the erythrocyte and hemoglobin counts

TABLE 1

	PER CUBIC MILLIMETRE	PER CENT
Erythrocytes	6,000,000	
Hemoglobin		95
Leucocytes	10,000 to 15,000	
Lymphocytes	5,000 to 8,000	35 to 55
Large mononuclears	500 to 1,000	5 to 8
Transitionals	0 to 300	0 to 3
Neutrophiles	5,000 to 10,000	35 to 60
Eosinophiles	300 to 400	3 to 4
Basophiles	100	1

in guinea pigs than in man. The absolute lymphocyte count in the guinea pig is about equal to the total leucocyte count in man. It is interesting to note that the newborn guinea pig has a red blood cell count of 7,000,000, hemoglobin of 100 per cent, and leucocyte count of from 3,000 to 8,000 with only about 25 per cent lymphocytes (see table 2). *Thus the lymphocyte picture in infancy and maturity is completely reversed in man compared with the guinea pig.* This seems to be an important fact in view of the studies on immunity and resistance to tuberculosis. Thus, it is believed, and some experiments above quoted indicate, that the lymphocytes are an important factor in resistance to tuberculosis. Nevertheless, it is well known that the human infant and the guinea pig show particularly slight resistance to the infection.

The variations in the total leucocyte count were usually due to the neutrophilic polymorphonuclear cells and in the extreme cases were directly traceable to acute infections, particularly infected wounds of the

back received in fights. These are well seen in no. 20, chart 2, in the late summer of 1921. It was necessary to kill two of the animals as a result of these injuries. The acute infections were accompanied by a temporary drop in the red blood cell count and hemoglobin, with the appearance of nucleated erythrocytes.

TABLE 2

Leucocyte counts of newborn guinea pigs

ANIMAL	W.B.C.	LYMPHOCYTES	LARGE MONO-NUCLEARS	NEUTROPHILES	EOSINOPHILES	KURLOFF BODIES
		per cent	*per cent*	*per cent*	*per cent*	
94b	800	50	5	43	0	0
95b	3,000	40	12	48	0	0
98b	3,000	13	4	81	0	0
99b	4,500	34	4	60	1	0
100b	4,000	16	8	70	3	0
101b	8,000	10	6	84	0	0
102b		25	10	58	6	0
103b		14	7	80	1	0

TABLE 3

Seasonal variations in the color index of normal guinea pigs

ANIMAL	PERCENTAGE COLOR INDEX FOR			
	April, 1921	April, 1922	August, 1921	August, 1922
1	98	96	94	90
2	97	93	93	
3	105	96	95	90
19	101		88	
20	100	97	93	95
45		97		90
46		96		90
Average.....	100	96.5	92.5	91
Seasonal average.....	98.3		91.8	

Seasonal difference = 6.5 per cent.

N.B.: 100 per cent represents 6,000,000 R.B.C. and 95 per cent Hb.

With the exception of these periods of acute infections, the red blood counts held a fairly even course. It will be noted that the charts were made on the basis that 6,000,000 red blood cells and 95 per cent hemoglobin is 100 per cent normal. However, the hemoglobin curve frequently falls below that of the red blood cell curve. A very interesting seasonal variation is seen in this drop in hemoglobin relative to the

corpuscular count. The hemoglobin curve most closely approaches or exceeds the red blood cell curve in winter or early spring, and the divergence of the two is greatest in late summer. In other words, the color index is lower in summer than in winter (see table 3). This is even more clearly brought out in the animals with chronic tuberculosis, which will be discussed later. Might this not represent a physiological variation in oxidation and heat production to accommodate the different seasons? Recently Hess and Lundagen (30) have reported seasonal tides in blood phosphates in infants, which they believe to be due to similar tides in ultraviolet rays of the sunlight, since the infants have more phosphates during the summer or when exposed to the direct rays of the sun. Our guinea pigs are never exposed to the direct rays of the sun, so it is doubtful whether the variations in hemoglobin content of the individual blood corpuscles could be dependent on this factor. But we agree with Hess and Lundagen that the seasonal variation in the blood and other tissue activities justifies much further study.

Kurloff bodies: The eosinophile count was uniformly low in the early months of the investigation, after which there was a sudden and marked rise which was maintained for the rest of the time. Coincident with this eosinophilia, the so called Kurloff bodies were first noted in the cytoplasm of some of the large mononuclear (indicated by crosses on the large mononuclear curves in the charts). It has been our intention to make a rather careful study of these bodies, since we believe that reservations must be made on all conclusions drawn from hematological studies in guinea pigs, until these bodies are explained, more particularly because of the coincident eosinophilia found in our normal animals.

Many theories have been offered as to the nature and significance of these Kurloff bodies but none have as yet been satisfactorily proved.

1: Kurloff (28), who first noted them in 1888, was of the opinion that they were aggregations of the punctate granules of the cytoplasm of lymphocytes, and that, although these granules are common to the lymphocytes of many species, the aggregation of them in the form of these so called Kurloff bodies is peculiar to the guinea pig and related families. This theory is also held by Pappenheim and Ferrata (31) and Maximow (personal interview).

2: Schilling-Torgau (32), Patella (33) and Ledingham (34) suggest that they may be cell secretions reacting against toxins, body hormones or immune bodies.

3: It was believed by Ledig, Goldhorn (35) and Hunter (36) that they are intracellular protozoa, parasites, or *Chlamydozoa.*

4: Ross (37) offers evidence to prove that they are *Lymphocytozoa cobayae,* or spirochetes, related to the virus of human syphilis, in which the Kurloff body represents the intracellular stage in the life cycle, and he claimed that spirochetes are to be found free in the blood stream.

5: Woodcock (38) maintains that they are incompletely digested, phagocytosed red blood cells.

A detailed description, with many excellent colored plates, is given by Pappenheim and Ferrata (31).

We have studied these Kurloff bodies in connection with our work but are as yet undecided as to their nature. These studies will be continued, but the following preliminary report may be made:

1. The Kurloff bodies did not appear in the early months of our work. Many of these early smears had been preserved and were carefully reëxamined after the Kurloff bodies had been studied, and we were able to verify the fact that they had not been merely unrecognized and overlooked in the first examination. They were first noticed in large numbers in the blood of the animals in one particular cage, and then seemed to spread to all the surrounding cages, until all animals were found to possess them. All animals that have entered our laboratory subsequent to that time have had Kurloff bodies. They were found in guinea pigs examined in three other laboratories of the University, although rats, mice, and rabbits living under the same conditions had no intracellular bodies. The German worker, Pappenheim (39), reported that all guinea pigs in his experience had Kurloff bodies. Maximow (personal interview) claims that all the guinea pigs in Russia investigated by him had them. Ross (37) stated that 90 per cent of the guinea pigs of England were infected with his so called *Lymphocytozoa cobayae.*

2. While there had been an eosinophile count in normal animals at a normal level, never to exceed 4 per cent, previous to the appearance of the Kurloff bodies, coincident with their appearance the eosinophile counts of these animals immediately increased, frequently reaching a 30 per cent level. None of the other workers have noted any relation between the Kurloff bodies and eosinophiles, although several report a marked variability in the eosinophile count in supposedly normal animals. Burnett (26) found the eosinophiles of 17 animals, some of which had intracellular bodies, to vary from 1 to 20 per cent, but he suggests no relationship between the two facts. Maximow recognizes the

variability of the eosinophiles in guinea pigs but does not believe that it is related to the Kurloff bodies.

3. We found, as did Pappenheim (39), that the embryo and newborn guinea pigs do not possess Kurloff bodies, even though the mother has them in large numbers. We could not verify the fact, as suggested by Pappenheim, that pregnant animals had more Kurloff bodies than other animals. The Kurloff bodies appeared in the young guinea pigs between the second and third months of extrauterine life. These young animals had been left in the same cages with animals possessing the bodies. So far, efforts to transfuse blood from animals known to possess the intracellular inclusions into these young guinea pigs have failed, due, apparently, to the incompatibility of the blood, as the animals died immediately in shock. This work will be repeated.

4. We have failed to confirm the work of Woodcock (38), who believes that the Kurloff bodies represent phagocytosed red blood cells which the lymphocyte is unable to digest completely. All the technique suggested by Woodcock has been used repeatedly, as have been many other staining methods, but no iron stain for the Kurloff body was successful, nor did any other stain show any marked resemblance between the red blood cell and the Kurloff body. A lymphocyte in the process of ingesting a red blood cell has never been seen.

5. We have also been unable to confirm the work of Ross (37), who stated that he saw free spirochetes in the blood of animals possessing Kurloff bodies, and could trace a direct relation between the two. We studied the blood of many animals, and of each animal several times, with the dark field illuminator, and also examined emulsions of the liver, spleen, bone-marrow and testes with the dark field illuminator. Smears of the blood and organs were also made and stained with the long Giemsa method and with India ink. But in no instance was a spirochete ever seen, or any other microörganism free in the blood stream or in the organ emulsions. However, many irregular small scars were seen in the liver of two of the normal animals (nos. 45 and 46). Microscopic studies revealed small areas of necrosis beneath the capsule, at points where the capsule was drawn in and the endothelial cells of the capsule had assumed a cuboidal shape. There was no fibrosis. Small areas of round cells appeared irregularly throughout the liver. They were not perivascular. Ross reported this condition also and thought the lesions were gummata. He also thought that a slight anemia was caused by the Kurloff bodies.

6. Blood smears containing Kurloff bodies and stained with Wright's and Giemsa stains were submitted to Dr. E. E. Tyzzer of Harvard University and Dr. B. H. Willier of the University of Chicago, and each stated independently that there was no definite evidence that the Kurloff body was any known parasite.

7. We have found that new methylene blue *G.G.*, used as a supravital stain, is specific for Kurloff bodies and is the best way of identifying them. They invariably take on a brilliant cherry red, while the cytoplasm and nucleus of the cell, containing the inclusion, and all other cells of the blood are blue, except that in certain concentrations a varying degree of a reddish hue may be acquired by the basophile granules which cannot be confused with a Kurloff body. Related dyes, such as capri blue and Nile blue, do not give the same results.

The following tentative conclusions may be drawn:

1. The nature and significance of the Kurloff body is still uncertain and the subject requires further study.

2. Their possible relationship to the variable eosinophile count makes it impossible to draw any conclusions about experimental eosinophilia in animals possessing Kurloff bodies.

3. They may possibly be associated with small, irregular scars in the liver and with a mild anemia, and thus help to confuse the picture of experimental tuberculosis.

STUDIES ON ANIMALS WITH ACUTE EXPERIMENTAL TUBERCULOSIS

An acute infection was developed in guinea pigs that were heavily inoculated. They usually died of generalized tuberculosis in less than four months. Autopsies of them revealed the usual picture of generalized tuberculosis of lymph nodes, spleen, lungs and liver. There was a rapid loss of weight before death. Fourteen animals of this type were examined after inoculation until death. One hundred and forty complete counts were made (see nos. 6 and 26, chart 1, 28, 29, and 36, chart 2 and chart 3). Charts of these examinations show very typical curves for the blood in acute generalized experimental tuberculosis. The anemia progresses rapidly, the erythrocytes frequently decreasing steadily, with relatively few variations, to 3,500,000. Nucleated erythrocytes were seen only three times, and then not to exceed 3 per 100 leucocytes. The hemoglobin dropped much more rapidly than the red blood cells,

falling at times to 35 per cent. The leucocyte count was more variable, but the greatest variations were due to the neutrophilic polymorphonuclear leucocytes. At termination, either a leucocytosis or a leucopenia might be shown. The leucopenia, when present, was more marked than the leucocytosis; counts below 7,000 being seen in animals 23, 26, 28 and 38. The leucocytosis might be considered as signifying a terminal secondary infection, but there was no demonstrable pathological difference between the animals dying with leucopenia and those dying with leucocytosis. Even when the leucocytosis was between 20,000 and 30,000, as in animals, 5, 7, 35, 36 and 39, it was entirely due to the neutrophilic polymorphonuclear cells.

The lymphocytes persistently decreased until death, and there was never any indication of an increased lymphocyte count; at death they were frequently one-fifth to one-tenth that of normal animals. A preterminal increase of lymphocytes in one animal, to almost 8,000 is an exception that we cannot explain. At any rate, it does not represent, apparently, an increased resistance. The large mononuclear and transitional cells show a preterminal increase to about double. They may stay at this level until death, or again decrease to normal or below. McJunkin (24) recognized such an increase in what he called endothelial cells, and thought they might indicate a last desperate effort on the part of the body to develop a resistance to the disease, but he was unable to demonstrate any increased resistance to tuberculosis in animals in which he induced an artificial endothelial leucocytosis. We are not sure that our large mononuclear and transitional cells are all identical with McJunkin's endothelial cells, although Sabin (40) has recently brought forward evidence to show that what are generally large mononuclear and transitional cells are of endothelial origin. There was no evidence in our work that this late increase in the large mononuclear elements increased the resistance of the animal. On the other hand, it might be suggested that their increase represents a final breakdown of the body, since death follows always within a few days, and that they may arise from the endothelium of the badly injured spleen and liver.

Eosinophiles were decreased or absent in all but one case (no. 36, chart 2). This animal had an eosinophilia of from 4 per cent to 7 per cent and lived longer than any other acute case. Kurloff bodies were never noted in any of these cases.

When the guinea pig died of some acute infection, such as peritonitis, the blood picture looked as though it had been left uncompleted, when compared to that of animals that died of generalized tuberculosis.

Chart 3 is of particular interest, as it shows 3 guinea pigs in presumably the same condition, given the same inoculation of the tubercle bacilli at the same time and yet with very different blood pictures. Case 5 really belongs to the type of chronic tuberculosis and will be discussed with the other chronic cases. This chart shows that the more rapid and acute the tuberculous process the more marked are the blood changes. This is particularly true of the tuberculous anemia, that is, the decreases in the red blood cells and their hemoglobin content, and of the lymphocytes. Autopsies showed that the animal which died first had the most extensive tuberculous lesions and the one that died last the least.

It may be concluded that in acute experimental tuberculosis of the guinea pig, a prognosis of the disease may be fairly easily made from the progress of the blood picture, unless the life of the animal is cut short by some acute secondary infection.

STUDIES ON ANIMALS WITH CHRONIC EXPERIMENTAL TUBERCULOSIS

A chronic infection developed in animals which received relatively light inoculations and showed a prolonged resistance to the disease (see nos. 8, chart 1, 14, chart 2 and 5, chart 3). These animals lived nine months or more after inoculation and frequently died of conditions other than tuberculosis, especially pneumonia, and never showed at autopsy tuberculous lesions as extensive as those in the acute cases. In some cases, in fact, no macroscopic lesions were found at death twelve to fourteen months after inoculation, although enlarged regional lymph nodes had at various times been palpated and these had later discharged through ulcers at the point of inoculation; also with these the blood pictures varied considerably from those of the uninoculated animals, resembling those of the other animals with chronic tuberculosis.

Thirteen animals with chronic tuberculosis were examined, a total of 350 complete blood counts being made. The most striking phenomena in the blood charts of chronic tuberculosis are the marked fluctuations of all the elements. These fluctuations are seen in the erythrocytes and their hemoglobin content, the total leucocyte counts and the individual leucocyte cells, such as the neutrophiles, lymphocytes and eosinophiles. There seems to be a tendency for all the elements to fluctuate together in the larger variations, although smaller variations in the individual elements occur by themselves. There are also undoubtedly other factors at work on the blood picture, such as leucocytic reactions to secondary infections, eosinophilia, etc. But, allowing for such factors, the blood

picture suggests the possibility that the variations are not due to absolute increases and decreases in the cells of the blood, but to relative changes due to variations in the blood concentration or plasma content of the blood. As the guinea pigs did not have hemoptysis, night sweats or diarrhea, this apparent change in blood concentration cannot be caused by these factors, as assumed in clinical work, but must be related to some such factor as that suggested by the work of Gaertner and Roemer (18), who showed that the products of tubercle bacilli cause an increased lymph flow in dogs. We believe this marked fluctuation to be the most significant part of the blood picture in chronic tuberculosis, and one that demands further study and consideration, not only to determine the underlying causes, whether these be a secretagogue action of the products of the infection or lesions, changes in the permeability of the blood capillaries, or changes in the osmotic pressure of the blood; but also because the fluctuation explains the great diversity of opinion as to the blood changes that occur in tuberculosis, especially when conclusions are drawn from one count or a few isolated counts on each patient; and it also indicates the futility of attempting to make a diagnosis from one blood count.

We are now engaged in carrying on some experiments, adapted to the living guinea pig, on the variability of the blood concentration of similar tuberculous animals.

A more careful analysis of the charts of these chronic tuberculous animals brings out certain other points:

1. The animals never showed the blood picture characteristic of acute tuberculosis, not even at termination. It is just as likely that the animal will die at a high peak of cellular count, including erythrocytes and hemoglobin (see nos. 5 and 8).

2. There is a characteristic chlorotoid anemia, not, however, as marked as that seen toward the end in acute tuberculosis. This is apparently not progressive, for if allowances be made for the seasonal variations in the hemoglobin content of the red blood cells, it will be seen that after the first few months of the disease the erythrocyte curve and the hemoglobin curve are roughly parallel. The seasonal variation is clearly seen, however (see table 4). Thus, the seasonal difference in the color index between April and August averaged 10.3 per cent for chronic tuberculosis, as compared to 6.5 per cent for normal animals. Nucleated erythrocytes were never seen.

3. The least variations are seen in the mononuclear elements. There is not a progressive decrease in the lymphocytes as seen in the acute cases; the lymphocytes were never unusually low, even as the end approached, with the autopsy indicating death from tuberculosis. On the other hand, with the exception of the one isolated count of case 14, the lymphocytes never exceeded the normal absolute value. There was never a temporary or prolonged lymphocytosis, even in animals living almost two years after inoculation and then very few tuberculous lesions. We thus obtained no evidence to indicate that resistance to tuberculosis is maintained by the lymphocytes. Of course, it is possible that the more circumscribed lesion of the reinfections

TABLE 4

Seasonal variations in the color index of guinea pigs with chronic tuberculosis

ANIMAL	PERCENTAGE COLOR INDEX FOR			
	April, 1921	April, 1922	August, 1921	August, 1922
7	103	101	83	82
8	95	92	88	77
9	99	94	77	86
10	85	88	82	89
11	96		82	
12	103	93	85	86
14	87	85	83	
Average.....	95.4	92.2	82.9	84
Seasonal average.....	93.8		83.5	

Seasonal difference = 10.3 per cent.

N.B.: 100 per cent represents 6,000,000 R.B.C. and 95 per cent Hb.

of adult human tuberculosis stimulates the production of a lymphocytosis, when the amount of systemic intoxication is undoubtedly much smaller than that of the marked generalized tuberculosis of our acutely infected, and probably even of the chronic infected, nonsensitized guinea pigs, in which the lesions tend to be destructive rather than proliferative. Nevertheless, it is interesting to note W. J. Mayo's (41) recent suggestion that the lymphatic system is a point of weakness in the body, in which such diseases as cancer, syphilis, and tuberculosis are concerned, rather than a point of strength as is usually assumed. Attention should again be called to the fact that the guinea pig, like the human infant, has a very high percentage of lymphocytes and a relatively low resistance to tuberculous infection. It would be worth while to determine the resistance of the

newborn guinea pig, which has a low lymphocyte count similar to that of the human adult. This has apparently not been done. Experiments (42) indicating that stimulation of lymphoid tissue increases resistance to tuberculosis and vice versa (21) have been questioned by more recent work (22). While the assumption that resistance to clinical tuberculosis is associated with a lymphocytosis is based on such work as that of Webb, Gilbert and Newman (43), Cummings (15), who studied the lymphocytes in 100 patients with tuberculosis and in 40 normal individuals, found an average of 30.5 per cent lymphocytes for the normal individuals, 27.4 per cent for tuberculous cases of good prognosis, and 16 per cent for those of bad prognosis and believed that "a high percentage of lymphocytes was indicative of increased resistance." But even in those cases of good prognosis the percentage is below normal. Moreover, these are percentage and not absolute figures, and their value depends largely upon the polymorphonuclear counts which vary more markedly in open cases of pulmonary tuberculosis than do the lymphocytes.

4. The eosinophiles in all our chronic cases held a high level (except for case 5). As the animals of one group had all been studied and had died before we first observed Kurloff bodies, we were of the opinion that this marked eosinophilia was an indication of increased resistance to the infection, more especially as neither the normal animals nor the acute cases examined over the same period showed any eosinophilia. However, all following cases showed both Kurloff bodies and eosinophilia, and the tracings were very similar to those of the normal animals after Kurloff bodies were noticed in their blood. Therefore, no certain conclusions about the eosinophilia can be drawn at present.

Thus, the blood picture in chronic tuberculosis is characterized by marked fluctuations in all elements, a nonprogressive chlorotoid anemia, an absence in lymphocytic reaction and a possible eosinophilia.

COAGULATION TIME

Tests of the coagulability of the blood were not made in the first part of the investigation. They were begun in November, 1921 (see chart 4). The Boggs coagulometer was employed. A total of 200 tests were made, 70 on 5 normal animals, 30 in 5 cases of acute tuberculosis, and 100 in 7 cases of chronic tuberculosis. An average time of about five minutes, with variations between four and five and one-half minutes, was obtained for the normal animals. This decreased a little, to about four or four and

one-half minutes, as the guinea pigs increased in age. The animals with chronic tuberculosis showed very little variation from this; although the progressive shortening of the time with increasing age seemed a little more marked, falling to about three and one-half minutes. But a very marked decrease in coagulability was noted in the animals with acute tuberculosis, the time being about doubled just previous to death. This may be associated with the marked liver injury which was always present in such cases. It is interesting, in this connection, to find that the doubling of the number of endothelial cells, before mentioned, occurs at the same time with the high peak of the coagulation time, while both phenomena are absent in the chronic cases.

No previous work on the coagulation time in experimental tuberculosis has been reported. For human tuberculosis Galera (44) records a retarded coagulation, due, he believed, to the scarcity of blood calcium. Solis-Cohen (45) reports a shortened clotting time associated with hemoptysis. Burns and Young (46) found no striking relation between the coagulation time and hemoptysis. Rudolph and Cole (47) found the average normal time to be seven minutes, while the average time for 9 cases in all stages of pulmonary tuberculosis was 8.9 minutes. Halverson, *et al.*, (48), showed that there is no marked deviation in the calcium content of the blood in patients in the various stages of tuberculosis. The guinea pigs did not have hemoptysis; so the cause for the retardation in the coagulability in our acute cases must be sought for elsewhere, possibly in the hepatic lesions.

ARNETH COUNTS

Sixty-four Arneth counts were made on 28 animals; 12 counts being made on 7 normal animals, 18 counts on 10 acute cases of tuberculosis, and 34 counts on 11 chronic cases (see table 5). When starting these examinations we were unable to find any references to previous work on the Arneth count in experimental tuberculosis, and in studying the blood of normal guinea pigs, compared to normal human blood, the conclusion was drawn that the leucocytes of the guinea pig had a relatively larger number of nuclear parts than the leucocytes of man. Thus, 7 groups were made and the majority of cells were thought to belong to groups 4 and 5, while Arneth (49) placed human blood in 5 groups with the majority of cells in groups 2 and 3.

TABLE 5

Arneth index

ANIMAL	DATE OF EXAMINATION	NUMBER OF NUCLEAR PARTS PER 100 CELLS	NUMBER OF NEUTROPHILES PER CUBIC MILLIMETRE OF BLOOD	DATE OF DEATH
		Normal animals		
1	2/13/22	443	4,800	
2	8/29/21	417	6,200	
3	10/ 8/21	401	6,100	
	4/15/22	422	2,900	
	5/17/22	454	3,700	
20	2/13/22	437	9,200	
19	8/22/21	336 (Acute infection)	1,900	
45	1/13/22	489	6,000	
	2/14/22	437	6,700	
	3/22/22	476	6,200	
46	2/13/22	502	6,700	
	3/13/22	504	4,600	
Average.....		448	6,300	
		Chronic tuberculosis		
5	9/22/21	414	4,100	
	10/10/21	334	5,800	1/ 2/22
7	2/ 8/22	422	10,300	
	3/ 9/22	415	6,200	
	5/29/22	439	7,800	
	8/ 2/22	421	7,300	9/ 5/22
8	1/ 6/22	431	5,100	
	2/ 8/22	412	8,400	
	3/ 9/22	419	6,200	
	4/ 5/22	404	10,100	
	5/29/22	422	7,000	
	10/12/22	412	10,700	10/18/22
9	2/ 8/22	444	8,800	
	3/ 5/22	427	13,600	
	8/ 3/22	461	6,900	
	10/11/22	462	12,800	
10	2/10/22	416	7,400	
	3/10/22	484	8,000	
	5/17/22	472	7,000	12/25/22
11	2/ 8/22	474	8,300	
	3/11/22	427	4,100	4/14/22
12	3/13/22	426	10,700	
	10/12/22	434	5,900	11/25/22
14	8/18/21	400	4,800	
	2/11/22	447	18,600	
	3/22/22	424	15,100	

TABLE 5—*Continued*

ANIMAL	DATE OF EXAMINATION	NUMBER OF NUCLEAR PARTS PER 100 CELLS	NUMBER OF NEUTROPHILES PER CUBIC MILLIMETRE OF BLOOD	DATE OF DEATH
		Chronic tuberculosis—Continued		
	5/16/22	402	15,400	
	6/22/21	325	8,200	7/26/22
17	8/23/21	402	13,800	
	9/21/21	409	22,800	11/ 2/21
41	10/10/22	422	6,200	
42	3/ 7/22	450	6,000	
	8/23/22	421	7,400	
	10/10/22	433	5,500	12/23/22
Average.....		426	9,000	
		Acute tuberculosis		
6	7/19/21	406	11,800	8/12/21
23	8/11/21	328	4,600	8/11/21
26	8/31/21	329	3,600	
	9/ 5/21	276	1,100	9/ 7/21
27	7/27/21	350	7,000	
	7/28/21	363	7,200	7/28/21
28	8/11/21	316	2,300	8/11/21
29	8/19/21	387	5,400	
	9/27/21	355	7,400	10/ 3/22
31	8/31/21	405	5,800	
	10/ 7/21	405	14,400	10/17/22
36	1/28/22	401	15,400	
	3/ 2/22	406	13,300	
	3/10/22	424	17,700	3/13/22
40	4/ 1/22	409	4,800	
	5/ 1/22	389	6,700	
	6/ 7/22	377	4,500	6/23/22
14	6/22/22	325	8,200	7/26/22
Average.....		342	7,700	

After completing our work we found a paper by Treadgold (50). He had determined a normal standard for guinea pigs on apparently the same basis as Arneth's work on man. In our work we have used the method suggested by von Bonsdorf (51), of adding all the nuclear parts of 100 polymorphonuclear cells together, and thus obtaining one figure representing the total nuclear parts per 100 leucocytes. This is much simpler than the Arneth-index picture. Thus, a decrease in nuclear parts per 100 cells represents Arneth's "shift to the left." Treadgold

also expressed his results in nuclear parts per 100 cells, and determined an average number of 267 in 5 normal guinea pigs while our average figure was 448. This indicates one of the greatest disadvantages of the Arneth count, which is the inability to standardize the counts, as no two persons interpret the nuclear parts in the same way. Treadgold then followed the process of tuberculosis in 3 inoculated guinea pigs and found a primary "shift to the right," or increase in nuclear parts, lasting from one to ten days and followed by a "shift to the left," or progressive decrease in nuclear parts, until death, when the indices were 204, 181 and 174. The table of our results shows an average of 448 nuclear parts per 100 cells in normal animals, of 426 in chronic tuberculosis, and 342 in acute tuberculosis. This decrease represents a "shift to the left." There are, however, certain conspicuous inconsistencies in the Arneth counts that are not easy to explain. Thus, about one-half of the normal counts are below the average for chronic tuberculosis, and about one-sixth of the chronic cases are above the average for normal animals. These latter might be explained on the basis of the "shift to the right" observed by Treadgold in his tuberculous animals. One of the normal animals had an acute secondary infection at the time the low Arneth count was observed, but the other cases are not so easily accounted for.

It was thought by Arneth that the "shift to the left" indicated that there was an increased demand on the bone-marrow for leucocytes, so that they were rushed into the blood stream before they were fully matured, and that the cells with the small number of nuclear parts represent young and inefficient cells. From our table it appears that this idea is borne out in part. Thus, the normal animals had the smallest number of neutrophilic polymorphonuclear cells per unit of blood and the largest number of nuclear parts per 100 cells, and in tuberculosis the number of cells increased while their nuclear parts decreased. However, the number of cells is less in the acute than in the chronic cases, while the nuclear parts are still decreasing. If, however, the Arneth counts are placed in three groups, based entirely on the value of the number of nuclear parts per 100 cells, we find that the average number of neutrophilic cells per unit of blood, in specimens having less than 350 nuclear parts per 100 cells, is 5,700; while there was an average of 6,400 neutrophiles in specimens having from 350 to 400 nuclear parts per 100 cells and an average of 9,300 neutrophiles in specimens having above 400 nuclear parts per 100 cells. This shows, then, that in our animals the large number and not the reduced number of nuclear parts is present in a leucocytosis.

An interesting relation exists between the anemia of the animal and the Arneth index. This is illustrated in chart 5, of case 14, which shows that the number of neutrophilic cells per unit of blood, the number of nuclear parts per 100 cells and the percentage of hemoglobin tend to rise and fall together.

CONCLUSIONS

1. Normal guinea pigs have a very uniform blood picture, particularly characterized by a seasonal variation of the hemoglobin content of the red blood cells, a high lymphocyte count and a high eosinophile count coincident with the appearance of Kurloff bodies. The newborn guinea pig has a low lymphocyte count.

2. An acute infection with tuberculosis produces a characteristic blood picture, in which there is a rapid loss in erythrocytes with a relatively greater decrease in hemoglobin, a terminal leucocytosis or leucopenia, a progressive decrease in lymphocytes, a marked preterminal increase in endothelial cells and an absence of eosinophiles.

3. Chronic tuberculosis produces a blood picture quite different from that of acute tuberculosis. It is characterized by a marked fluctuation in all elements, a relatively low hemoglobin content of the erythrocytes, which is not progressive but shows the normal seasonal variations, an absence of any reaction in the lymphocytes, and an eosinophilia which cannot, however, be distinguished from that associated with Kurloff bodies.

4. The nature of the Kurloff bodies and their effects on the guinea pig are still undertermined, and, until they are better understood, definite conclusions cannot be drawn from hematological studies on the guinea pig.

5. The coagulability of the blood in chronic tuberculosis shows slight changes from the normal, but in acute tuberculosis it is markedly retarded.

6. The Arneth counts show a decrease in nuclear parts per 100 cells in tuberculosis, which, on the average, is greater in acute than in chronic cases but there are individual exceptions.

REFERENCES

(1) Ullom and Craig: Am. J. M. Sci., 1905, clvi, 386.
(2) Klebs and Klebs: Trans. Nat. Assoc. Study & Prev. Tuberc., 1906, ii, 353.
(3) Sokolowski: Klinik f. Brustkrankh., 1906, ii. (Quoted by Klebs, Tuberculosis, 1909, p. 287.)
(4) Appelbaum: Berl. klin. Wchnschr., 1902, xxxix, 7.
(5) Grawitz: Ibid., 1895, xxxii, 713, 740.

(6) EWING: Clinical pathology of blood, 1903, p. 319.
(7) STRAUSS AND ROHNSTEIN: Die Blutzusammensetzung bei den verschiedenen Anämien, 1901.
(8) ZAPPERT: Ztschr. f. klin. Med., 1893, xxiii, 227.
(9) HALBORN: Rév. d. l. Tuberc., 1903, x, 319.
(10) SWAN: J. Am. M. Ass., 1904, xlii, 696.
(11) SOLIS-COHEN AND STRICKLER: Am. J. M. Sci., 1911, cxlii, 691.
(12) WEIL: Ztschr. f. Tuberk., 1918, xxix, 193.
(13) YOKOYAMA: Japan Med. World, 1922, ii, 261.
(14) MCDOUGAL: Brit. J. Tuberc., 1916, x, 159.
(15) CUMMINGS: Interstate M. J., 1916, xxiii, 12.
(16) ACHARD AND LOEPER: Compt. Rend. Soc. d. l. Biol., 1901, liii, 278.
(17) CLAUDE AND ALY-ZAKY: Gaz. Hebd. d. Méd. e. d. Chir., May 15, 1902.
(18) GAERTNER AND ROEMER: Wien. med. Bl., 1891, xiv, 654.
(19) HUSSEY: J. Exper. Med., 1921, xxxiii, 337.
(20) ROGERS: Tr. Nat. Tuberc. Ass., 1920, xvi, 363.
(21) MURPHY AND STURM: J. Exper. Med., 1919, xxix, 35.
(22) CORPER AND GAUSS: Tr. Nat. Tuberc. Ass., 1920, xvi, 354.
(23) TAYLOR AND MURPHY: J. Exper. Med., 1917, xxv, 609.
(24) MCJUNKIN: Arch. Int. Med., 1919, xxiv, 295.
(25) BETHE: Morphologisch. Arbeiten, 1892, i, 207.
(26) BURNETT: J. Med. Research, 1904, xi, 537.
(27) HAYEM: Du sang et de ses alterations anatomiques, 1889, p. 169.
(28) KURLOFF: cited by Ehrlich and Lazarus, Die Anamie, Nothnagel's Specielle Path. und Ther., 1898, viii, p. 56.
(29) COHENSTEIN AND ZUNTZ: Archiv. f. gesamt. Physiol., 1884, xxxiv, 173.
(30) HESS AND LUNDAGEN: J. Am. M. Ass., 1922, lxxix, 2210.
(31) PAPPENHEIM AND FERRATA: Folia Hematolog., 1910, x, 155.
(32) SCHILLING-TORGAU: Ibid., 1914, xvii, 443.
(33) PATELLA: Berl. klin. Wchnschr., 1908, xlv, 1846.
(34) LEDINGHAM: Lancet, June, 1906, i, 1675.
(35) GOLDHORN: N. Y. Path. Soc. Proc., 1905. (Quoted by Ross: Proc. Roy. Soc., of London, 1912, lxxxv, 67.)
(36) HUNTER: Univ. Penn. M. Bull., 1909, xxii, 333.
(37) Ross: Brit. M. J., December 14, 1912, p. 1651, and Proc. Royal Soc. London, 1912, lxxxv, 67.
(38) WOODCOCK: J. Royal Army Med. Corps, 1921, xxxviii, 321 and 418.
(39) PAPPENHEIM: Folia Hematolog., 1914, xvii, 183.
(40) SABIN: Physiolog. Rev., 1922, ii, 38.
(41) MAYO: J. Am. M. Ass. 1923, lxxx, 221.
(42) TAYLOR AND MURPHY: J. Exper. Med., 1917, xxv, 609.
(43) WEBB, ET AL: Amer. Rev. Tuberc., 1923, vi, 1073.
(44) GALENA: Riforma Med., 1921, xxxvii, 149.
(45) SOLIS-COHEN: Arch. Int. Med., 1911, viii, 820.
(46) BURNS AND YOUNG: Am. J. M. Sci., 1917, cliv, 800.
(47) RUDOLPH AND COLE: Ibid., 1911, cxlii, 483.
(48) HALVERSON, ET AL: J. Am. M. Ass., 1917, lxviii, 1309.
(49) ARNETH: München. med. Wchnschr., 1904, li, 1096.
(50) TREADGOLD: Lancet, 1920, i, 499.
(51) VON BORNSDORF: Beitr. z. Klin. d. Tuberk., 1913, v, suppl.

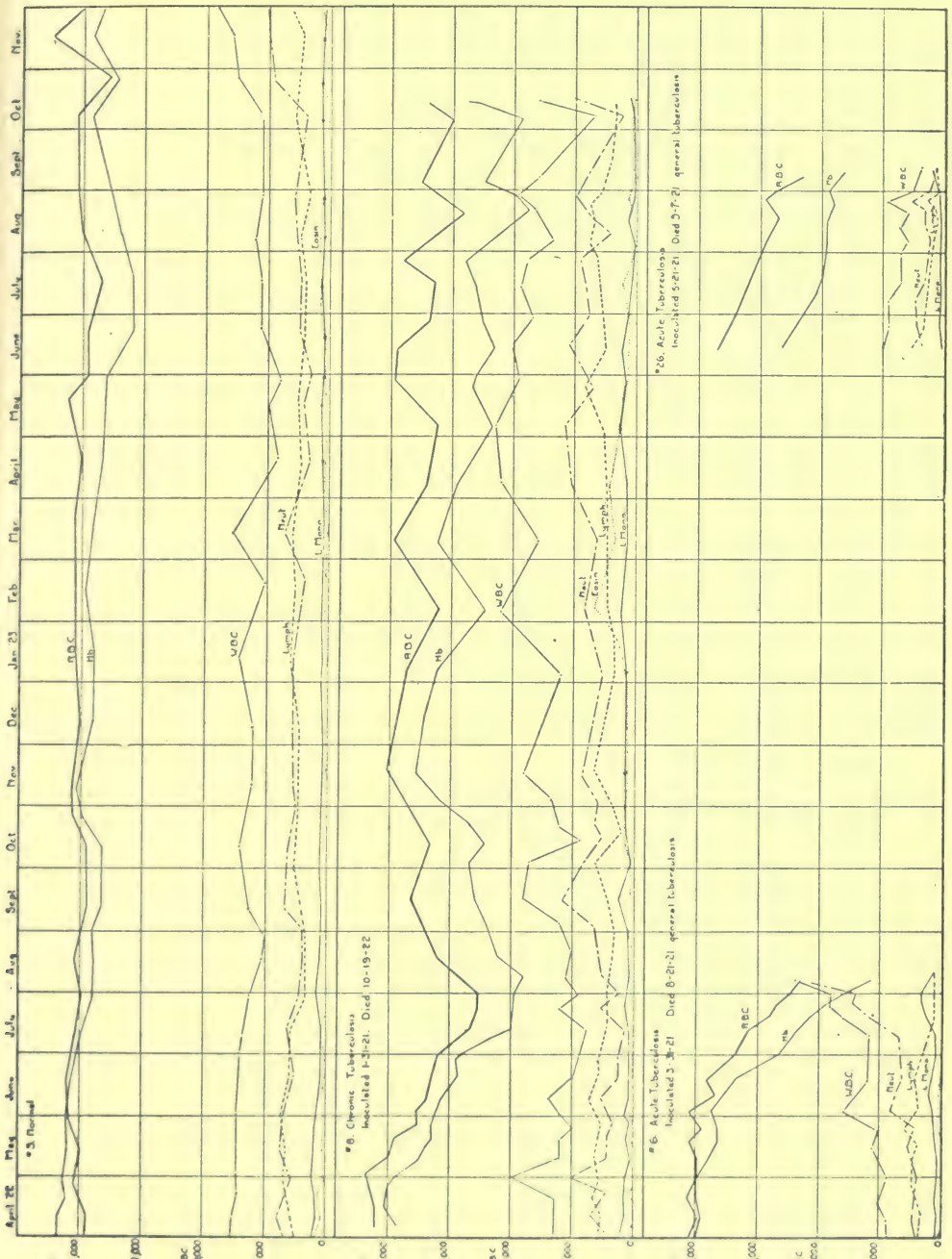
April 20 May June July Aug Sept Oct Nov Dec Jan 23 Feb Mar April May June July Aug Sept Oct Nov
#3. Normal
#8. Chronic Tuberculosis
Inoculated 1-31-21. Died 10-19-22
#6. Acute Tuberculosis
Inoculated 3-31-21 Died 8-21-21 general tuberculosis
#26. Acute Tuberculosis
Inoculated 5-21-21 Died 5-7-21 general tuberculosis
RBC
Hb
WBC
Neut
Lymph
Eosin
L Mono
Hb RBC
WBC

CHART 1

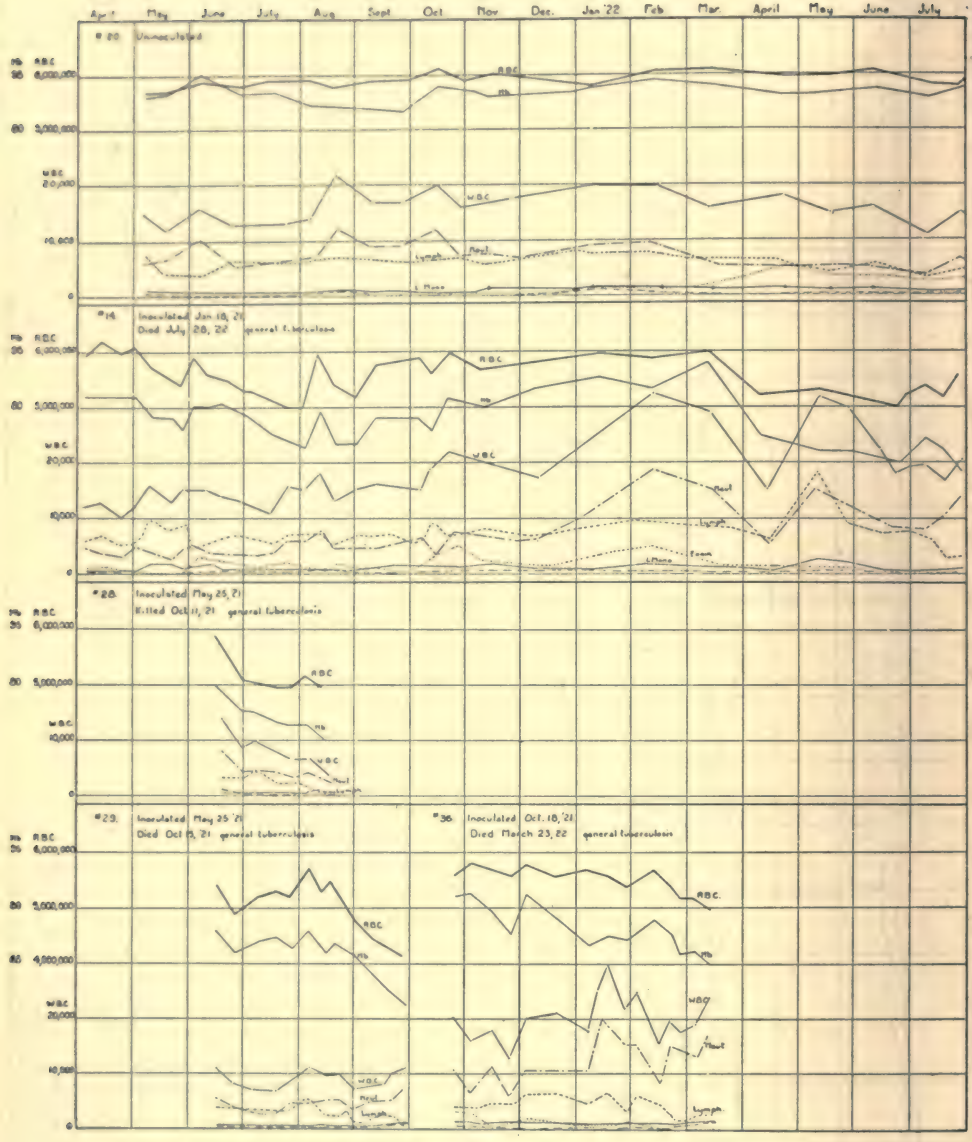
April
May
June
July
Aug.
Sept.
Oct.
Nov.
Dec.
Jan '22
Feb.
Mar.
April
May
June
July
#20 Uninoculated
#14. Inoculated Jan 18, 21
Died July 28, 22 general tuberculosis
#28. Inoculated May 25, 21
Killed Oct 11, 21 general tuberculosis
#29. Inoculated May 25 '21
Died Oct 13, '21 general tuberculosis
#36. Inoculated Oct. 18, '21
Died March 23, 22 general tuberculosis
RBC.
Hb
WBC.
Neut.
Lymph.
Mono.
Eosin.

CHART 2

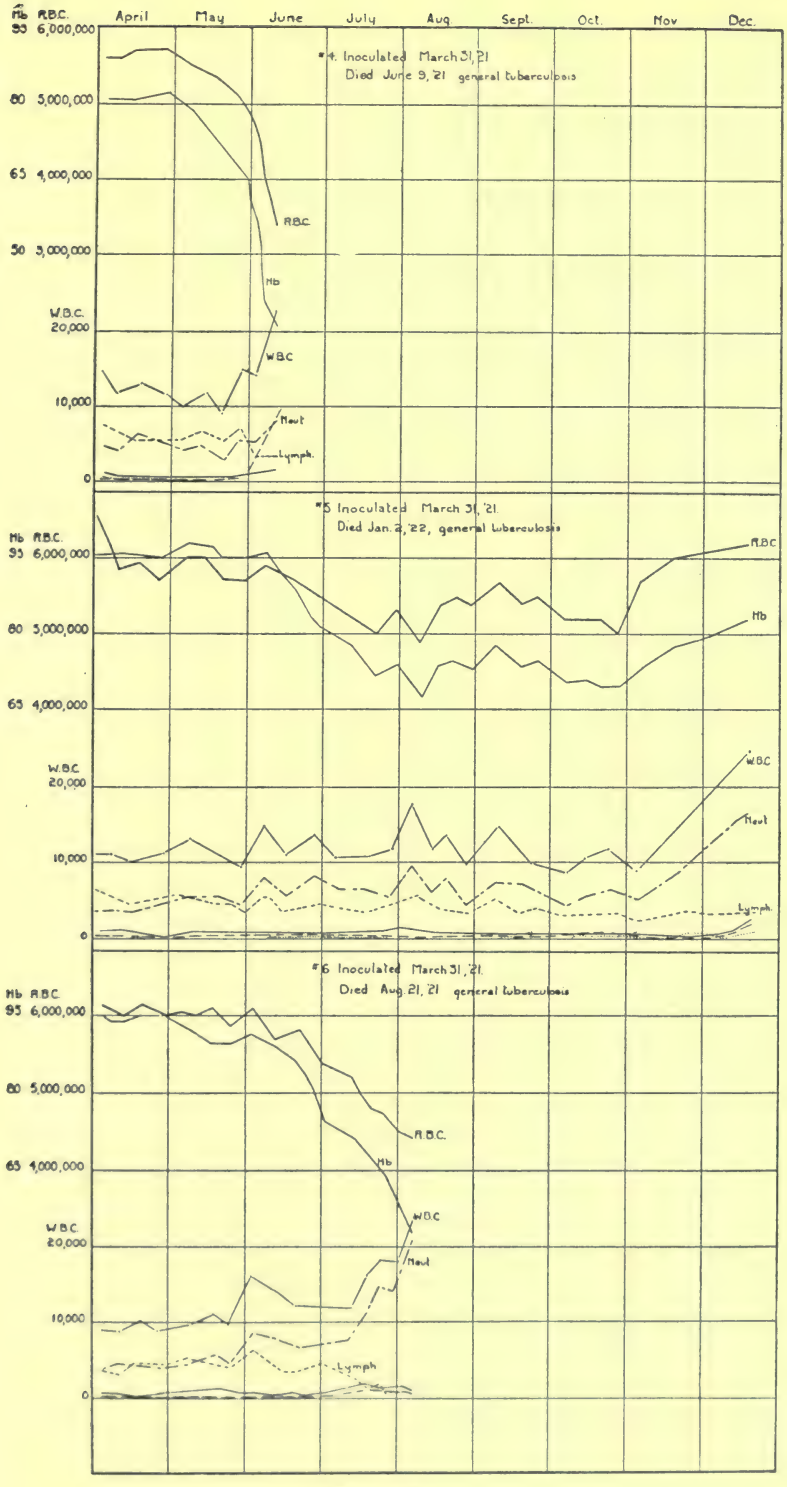
Hb R.B.C.
April
May
June
July
Aug.
Sept.
Oct.
Nov
Dec.
95 6,000,000
80 5,000,000
65 4,000,000
50 3,000,000
#4. Inoculated March 31, 21
Died June 9, 21 general tuberculosis
R.B.C.
Hb
W.B.C.
20,000
10,000
0
W.B.C
Mont
Lymph.
#5 Inoculated March 31, '21.
Died Jan. 2, '22, general tuberculosis
#6 Inoculated March 31, '21.
Died Aug. 21, 21 general tuberculosis

CHART 3

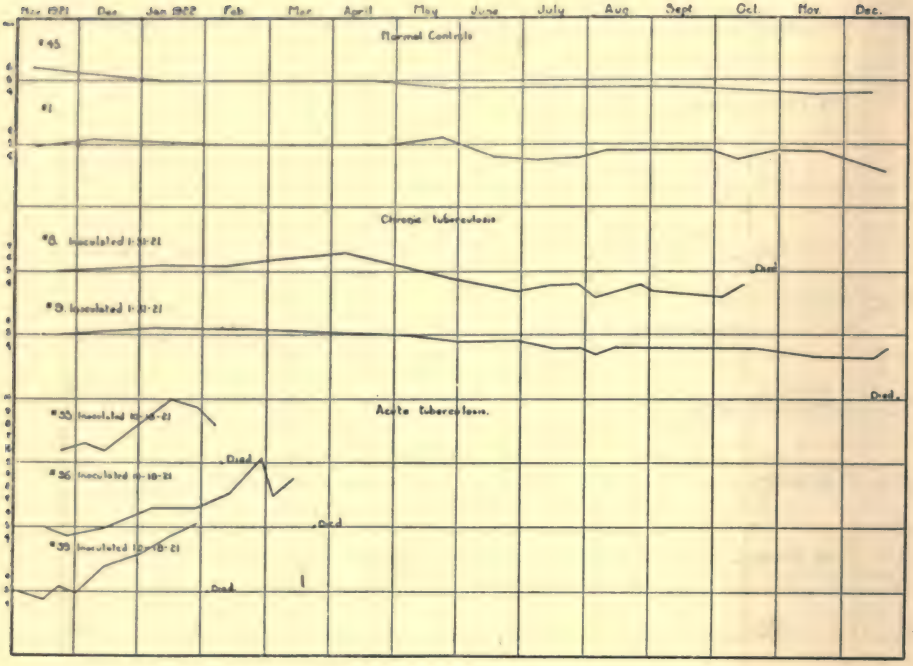

CHART 4. COAGULATION TIME OF BLOOD

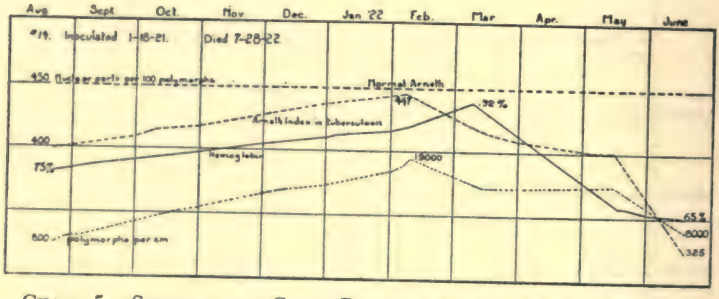

CHART 5. SHOWING THE CLOSE RELATION BETWEEN ARNETH INDEX, ANEMIA AND LEUCOCYTOSIS

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THE THERAPEUTIC AND BACTERICIDAL VALUE OF ORGANIC MERCURIAL COMPOUNDS IN EXPERIMENTAL TUBERCULOSIS IN GUINEA PIGS[1]

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. XXVIII

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In 1921 I published a report of my earlier work on over twenty compounds of mercury and their value in the chemotherapy of experimental tuberculosis (1). In this report the earlier literature on the subject was quite exhaustively reviewed. Any further review of the early literature on mercury seems unnecessary at this time.

Most of the compounds used in my earlier work were commercial preparations, bought on the market, some being inorganic and a few organic. A few were double salts of mercury with a dye, which were made for me by my chemical assistants after published methods. For some years before 1921 and ever since that time, several chemical assistants have been investigating for me the problem of making organic mercury compounds, with the special purpose of combining mercury with methylene blue to make a stable soluble compound, which should be more efficient than the double salts of methylene blue and mercuric chloride reported in the article referred to above. In connection with this investigation a considerable number of organic compounds of mercury with various compounds of phenol and of aniline were made. The methods of making these compounds have been described by them in the chemical literature (2) and will not be given in this report. The inhibitory power of these compounds on the growth of human tubercle bacilli *in vitro* was first tested and the report of the results was published in 1922 (3). The inhibitory power of compounds having mercury in various positions in the benzene ring and also the effects of various groups substituted in different positions were compared. It was found that certain of these compounds completely inhibited growth of the

[1] From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, University of Chicago.

tubercle bacilli in dilutions up to 1 in 100,000, while phenol itself inhibited only at 1 to 1000 and mercuric chloride at 1 in 40,000. The compounds having high inhibitory power were selected for therapeutic treatment of tuberculous guinea pigs and also for bactericidal experiments.

In this way, seven compounds of phenol and six of aniline were chosen for the work to be reported in this paper. The bactericidal power of these compounds was tested in the following manner:

A suspension of a very virulent human tubercle bacillus, 4015, from H. J. Corper's laboratory, was made in physiological salt solution, containing in each cubic centimetre 1 mgm. moist weight of the culture of 4015 grown for three weeks on glycerol-agar. To 10 cc. of this suspension were added sufficient amounts of the compounds to be tested to make dilutions 1 in 1000, 1 in 10,000 and 1 in 100,000. After thorough shaking of each flask, they were put in the incubator at 37.5°C. for one hour and twenty-four hours. At the end of each such time, 1 cc. was withdrawn and added to 10 cc. of physiological salt solution and well shaken. One-half cubic centimetre of this dilution was then injected subcutaneously into each guinea pig, three animals being used for each time, each dilution and each compound. Hence each guinea pig received a dose of 0.05 mgm. of the culture of tubercle bacilli. Controls were made in the same way, physiological salt solution being used instead of the drug. Only five of the thirteen compounds selected for therapeutic use were tested for their tuberculocidal power.

Table 1 gives the names and chemical formulae of these compounds and the results of the experiment as to the distribution of the disease in the four organs most generally affected in the tuberculous guinea pig. Three pluses indicate involvement of the organ in all three animals tested, while minuses indicate no extension of the disease to that organ. From this table it may be seen that mercuric chloride, though much lower in inhibitory power than any of the organic mercurials listed in the table, showed slightly higher tuberculocidal power than any except 14 M, while number 20 and P-nitroso phenol O-mercuric chloride, caused considerable lowering of virulence, as indicated by the degree of distribution, even at 1 in 100,000. Only with P-nitroso phenol O-mercuric chloride did one hour's exposure affect the distribution of the infection even at a concentration of 1 in 1000. O-nitro phenol P-carbinol O-mercuric acetate, the inhibitory efficiency of which extended to 1 in 100,000, showed the least tuberculocidal action.

TABLE 1

COMPOUNDS	ORGANS	1 TO 1,000		1 TO 10,000		1 TO 100,000	
		1 hour	24 hours	1 hour	24 hours	1 hour	24 hours
$HgCl_2$	Lymph nodes	+++	−−−	+++	−−−	+++	+++
	Spleen	+++	−−−	+++	−−−	+++	+++
	Liver	+++	−−−	+++	−−−	+++	+++
	Lungs	+++	−−−	+++	−−−	+++	+++
14 M	Lymph nodes	+++	−−−	+++	−−−	+++	+++
	Spleen	+++	−−−	+++	−−−	+++	+++
	Liver	+++	−−−	+++	−−−	+++	+++
	Lungs	+++	−−−	+++	−−−	+++	+++
No. 20	Lymph nodes	+++	−−−	+++	+++	+++	+++
	Spleen	+++	−−−	+++	+−−	+++	+−−
	Liver	+++	−−−	+++	+−−	+++	−−−
	Lungs	+++	−−−	+++	+−−	+++	+−−
P-Nitroso Phenol O-Mercuric chloride	Lymph nodes	++−	−−−	+++	+−−	+++	+−−
	Spleen	+−−	−−−	+++	−−−	+++	+−−
	Liver	+−−	−−−	+++	−−−	+++	−−−
	Lungs	+−−	−−−	+++	−−−	+++	−−−
Na No. 15	Lymph nodes	+++	+−−	+++	+++	+++	+++
	Spleen	+++	+−−	+++	+++	+++	+++
	Liver	+++	−−−	+++	++−	+++	+−−
	Lungs	+++	+−−	+++	+++	+++	++−
O-Nitro phenol P-Carbinol O-mercuric acetate	Lymph nodes	+++	+++	+++	+++	+++	+++
	Spleen	+++	+++	+++	+++	+++	+++
	Liver	+++	+++	+++	+−−	+++	++−
	Lungs	+++	+++	+++	+++	+++	+++
Control	Lymph nodes	+++	+++				
	Spleen	+++	+++				
	Liver	+++	+++				
	Lungs	+++	+++				

THERAPEUTIC EXPERIMENTS

Series 1: The animals of this series were inoculated January 15, 1920, subcutaneously, with 0.05 mgm. of 1305, a strain of human tubercle bacilli of moderate virulence. Eight of the animals of the series were kept untreated as controls and the others were divided into sets, with 8 guinea pigs in each set, and treated with the organic mercury compounds named below. The guinea pigs were fed 1 to 2 mgm. per day, the feeding

and the dosage being regulated according to the weight of the animal which was taken once a week. They also received weekly subcutaneous injections for a time, but these caused severe infiltrations, necrosis and ulcerations, and were therefore discontinued.

The following mercurial compounds were used in treatment of the guinea pigs in this series:

(1) *Number 9:* Sodium P-nitrophenol O-mercuric chloride, an isomer of mercurophen, having 52.8 per cent, of mercury and inhibiting the growth of tubercle bacilli *in vitro* in dilutions up to 1 in 100,000. The animals of this set lived 52, 100, 120, 176, 187, 224 and 276 days, or an average of 162 days, and received 45, 86, 106, 200, 220, 297 and 372 mgm., or an average of 189.4 mgm. for each. In several of the animals death was due to an acute suppurating infection and in none was the tuberculous involvement very extensive. In all some lymph glands were slightly enlarged but not caseous. The involvement of the spleen, liver and lungs was very slight.

(2) *Number 15:* Mercuri-bis-P-nitrophenol. This contains 42 per cent of mercury which forms a bridge between two nitrophenol rings. It prevents the growth of tubercle bacilli *in vitro* in dilutions to 1 in 100,000, but failed to kill all the bacilli in twenty-four hours, even at 1 in 1000. The 8 guinea pigs died after 7, 25, 26, 31, 31, 68, 159 and 224 days, the first 5 dying of acute infections and showing no tuberculosis. The other three received 63, 180 and 400 mgm. of the compound and showed very little or no tuberculosis.

(3) Dimethyl aniline P-mercuric acetate, having 52.8 per cent mercury. The compound completely inhibits in dilutions to 1 in 20,000 to 1 in 40,000. The maximum duration of life was 117 days and the average 95 days. All showed extensive involvement of lymph nodes, liver, spleen and lungs. Apparently, there was no beneficial effect from treatment with this aniline mercury compound.

(4) Symmetrical P-mercury dimethyl aniline, containing 45.8 per cent of mercury which forms a bridge between two dimethyl aniline rings.

This position should, according to Abelin (4), render the compound less toxic than most of the other organic mercurials, although perhaps also less active. This compound was quite insoluble in water and the subcutaneous injection of it suspended in oil caused much infiltration, necrosis and ulceration. The animals lived 25, 119, 125, 146 and 150 days, or an average of 113 days, having received by mouth 24, 106, 112,

129 and 160 mgm. of the drug, or an average of 106 mgm. per animal. All the animals except the first showed marked tuberculous involvement of lymph nodes, spleen, liver and lungs.

The 8 untreated controls of this series lived 14, 66, 66, 207, 213, 270, 277 and 301 days, or an average of 155 days. The two which died first died of acute infections, with very little tuberculous involvement. All the others showed marked and extensive tuberculous involvement of lymph nodes, spleen, liver and lungs. Hence, the controls of this series lived as long as or longer than the treated animals, though some of the treated animals showed much less extensive distribution of the disease.

Series 2: November 1, 1920, a second series of guinea pigs was inoculated subcutaneously with 0.0002 mgm. of number 4015, isolated a few months earlier by Dr. Corper, and said by him to be of such virulence that 0.00001 mgm. caused a slowly progressive tuberculosis, generalizing after about three months. The dose used by me caused generalized infection in about five weeks and the untreated controls died after 8, 22, 45, 63, 70, 84, 84 and 112 days, all except the first two showing generalized infection, the lymph nodes, spleen, liver and lungs being usually involved. The other guinea pigs of this series were treated by daily feeding of pills, each containing from 1 to 2 mgm. of the following organic mercury compounds. Weights were taken once a week to determine the condition of the animals and their ability to continue the treatment.

(1) *Number 15:* 2, 2′ Mercuri-bis-P-nitrophenol. This compound had 42 per cent of mercury and inhibited growth in dilutions to 1 in 100,000. It killed all bacilli only in dilutions of 1 to 1000 at twenty-four hours. These animals died after 3, 60, 78, 86, 88 and 99 days. All except the first showed generalized tuberculosis, lymph nodes, spleen, liver and lungs being usually involved.

(2) Saligenin dimercuric acetate or phenol O-carbinol 2, 4 mercuric acetate, a compound made and kindly sent to me by Dr. Hirschfelder of the University of Minnesota, and containing 45.5 per cent of mercury. The animals treated with this compound died after 15, 24, 36, 36, 56, 60, 135 and 176 days, or an average of 67 days. They had received 12, 21, 26, 26, 37, 40, 116 and 151 mgm. All except the first two had generalized tuberculosis, although in most acute infections had been contributory factors in their death. However, there was no indication that this compound had had any beneficial effect on any of the animals.

(3) P-nitroso phenol O-mercuric chloride, having 55.9 per cent of mercury and inhibiting in dilutions up to 1 in 100,000. The animals treated with this compound lived 4, 35, 53, 57, 58, 59, 61 and 64 days, or an average of 49 days. While an acute intestinal infection contributed much as a cause of the early death, all except the first to die showed some tuberculosis, mostly generalized.

(4) *Number 26:* M-nitro dimethyl anilin P-mercuric acetate, which has 47.2 per cent mercury and inhibits up to 1 in 100,000. This set lived 20, 36, 45, 47, 59, 74, 113 and 263 days, and received 17, 25, 30, 33, 45, 58, 92 and 204 mgm. of the compound. All showed some tuberculosis and all except the first two showed it widely distributed.

(5) *Number 27:* Dimethyl O-nitro aniline P-mercuric acetate, has 47.2 per cent mercury and inhibits up to 1 in 100,000. The guinea pigs treated with this compound lived 30, 30, 32, 32, 44, 48, 92 and 98 days, and received 20, 22, 22, 33, 37, 75 and 81 mgm. of the drug. While the first four died from subcutaneous suppuration, intestinal infection or pneumonia and had very little tuberculosis, the others all had extensive involvement of glands, spleen, liver and lungs.

(6) *Number 29:* Monomethyl P-nitraniline O-mercuric acetate, has 48.8 per cent mercury and inhibits growth of tubercle bacilli *in vitro* up to 1 in 100,000. This set showed very marked intestinal affections and all died within 44 days, yet all showed some tuberculosis and in most cases it was fairly well generalized.

(7) *Number 14 M:* Mono-ethyl P-nitraniline O-mercuric acetate, has 47.2 per cent mercury, inhibits growth up to 1 in 100,000, and has stronger tuberculocidal action than any of the other organic mercurials and equal to that of mercuric chloride. The acute intestinal and pulmonary affections prevailed among these guinea pigs also, but all lived long enough to develop some generalized tuberculosis, though the maximum duration of life was 57 days.

(8) *Number 20:* 2, 2′ hydroxy 4-nitroso-mercury diphenyl, with mercury at 48. 2 per cent and forming a bridge between the two phenyl rings. This has an inhibitory power ranging from 1 in 100,000 to 1 in 300,000, but its tuberculocidal action was not so great as that of *number 14 M*. The animals treated with *number 20* lived 39, 39, 47, 48, 51, 57, 60 and 104 days, having received 34, 34, 38, 38, 41, 46, 49 and 87 mgm. of the compound. The first four showed slight tuberculosis of lymph nodes and spleen, while the last four showed marked tuberculosis of these and liver and lungs also.

Series 3: Series 3 was inoculated January 18, 1921, with 0.025 mgm. of 1305. The object of this third series was to test the efficiency of these compounds in slowly progressing chronic tuberculosis of guinea pigs, since it was noted in my earlier report (1) that some drugs had a much more pronounced effect in chronic than in acute tuberculosis, and that this chronic tuberculosis in guinea pigs resembled more nearly human tuberculosis than does the more usual acute form. The untreated controls of this series showed no generalized tuberculosis after 76 days, but after 98 days lymph nodes and spleen were involved. The animals dying after 451, 556 and 656 days showed very marked tuberculosis of lymph nodes, spleen, liver and lungs, while one which lived 706 days showed no tuberculosis and no scars of healed tuberculosis.

The animals were divided into sets, 6 guinea pigs to each set. One set was kept as controls, while the other sets were treated each with one of the following organic mercurial compounds. Each guinea pig was fed daily a bread pill containing 1 mgm. of the drug for 11 days, after which 2 mgm. were fed. In the earlier part of the experiment they were also anointed with an oil containing 2 per cent of the same drug. They were weighed once a week and, if the weight was found materially decreased, the feeding was stopped for a week and then resumed, but there was very little necessity for interrupting the treatment in this series. The urine of a number of the animals was collected at intervals and examined by the Elliott method (5) for mercury. It always gave a positive test, showing that the compound is taken up by the blood and excreted by the kidneys. Several treated animals that died after long treatment with these drugs had their organs examined qualitatively for mercury after administration of total amounts varying from 27 to 577 mgm. Spleen, liver, kidneys and lungs all generally showed from a trace to a very distinct reaction, though in no case was there a weighable amount. The amount was estimated at about 0.1 mgm. in the organs by comparison with a test made on the organs containing a known amount. The spleen seemed to contain the largest amount, though the kidney was a close second.

The compounds used were as follows:

(1) *Number 26:* M-nitro dimethyl anilin P-mercuric acetate. This inhibits growth of tubercle bacilli completely up to a dilution of 1 in 100,000. These lived 140, 186, 350, 433, 475 and 610 days after receiving 176, 222, 356, 573, 651 and 1176 mgm. of the drug. All of these except the one that died after 433 days showed some general tuberculous in-

volvement, though in most cases very slight. The one exception showed no tuberculosis whatever.

(2) *Number 27:* O-nitro dimethyl anilin P-mercuric acetate. These lived 31, 93, 132, 173, 336 and 512 days, having received 46, 124, 157, 205, 473 and 775 mgm. of *number 27*, in addition to the amount absorbed from the anointing with 2 per cent oil suspension. The first and third of the animals to die showed no tuberculosis except slightly involved regional lymph nodes. All the others showed greater or less involvement of lymph nodes, spleen, liver and lungs, the involvement being about the same as in the untreated controls, so that apparently there was no beneficial effect from treatment from this compound.

(3) *Number 29:* P-nitro monomethyl anilin O-mercuric acetate. These lived 38, 342, 384, 512, 704 and 755 days and received 54, 422, 493, 714, 1046 and 1132 mgm. of *number 29*. The first to die showed no tuberculosis, the second showed only slightly enlarged regional lymph nodes, the spleen, liver and lungs appearing normal. The next two showed marked generalized tuberculosis, while the last two, whose duration of life was in the neighborhood of two years, showed enlarged, hard fibrous lymph nodes, spleen slightly enlarged, but hard with fibrous scars, and liver and lungs dotted with a few small hard, necrotic scars. Two of this set had apparently advanced somewhat further toward healing than is common in guinea pigs.

(4) P-nitroso phenol O-mercuric chloride. The animals of this set lived 70, 90, 156, 247, 409 and 467 days and received 97, 131, 241, 381, 759 and 859 mgm. of the compound. The first three to die showed extensive generalization, the next showed no tuberculosis except in the regional lymph nodes which were enlarged but hard and fibrous, and the last to die showed considerable fibrosis of lymph nodes and tubercles in spleen, liver and lungs. Apparently there was more checking of progress and more healing at an earlier stage in this set than in the others I have described.

(5) *Number 20:* P-nitroso mercury diphenyl. This compound contains 48.2 per cent mercury and inhibits in dilutions up to 1 in 100,000. These animals lived 89, 91, 95, 420, 545 and 656 days and received 135, 137, 141, 535, 709 and 903 mgm. of the drug. Only the last two showed any evidence of fibrotic healing.

(6) Hirschfelder's nitrosaligenin or O-nitro phenol P-carbinol O-mercuric acetate. This compound prevented growth of tubercle bacilli in dilutions up to 1 in 100,000, but its tuberculocidal power was not great,

as not even 1 in 1000 showed any effect, even after exposure for twenty-four hours. The animals treated with this drug lived 95, 115, 280, 528, 552 and 680 days and received 121, 141, 306, 554, 578 and 706 mgm. All died of some infection other than tuberculosis and most had very little tuberculous involvement, although nearly all had at some time had enlarged tuberculous lymph nodes.

(7) *Number 15:* Na, 2, 2′ mercuri bis P-nitrophenol, caused complete inhibition of growth of human tubercle bacilli *in vitro* in dilutions up to 1 in 100,000. It had nearly complete tuberculocidal action at 1 in 1000 after twenty-four hours' exposure. The animals treated with this compound lived 42, 264, 526, 536 and 807 days, while one is still living at the time of writing after 816 days. They received 64, 452, 900, 910 and 1542 mgm. of the compound by mouth. All except one showed some generalized tuberculosis in lymph nodes, spleen, liver and lungs, though in most cases the involvement was slight. The two that lived the longest, two and a quarter years after inoculation, received a tuberculin test, May 1, 1923, the test being given on the eroded skin and also by injection into one testis. In both the test was negative. One of the guinea pigs which had been partially paralyzed and subject to convulsions during the last six weeks, died within fifteen hours after the tuberculin was administered. Many apparently entirely healed tubercles were found in enlarged lymph nodes, enlarged spleen, liver and lungs. Microscopic examination showed no caseation, completely fibrous tubercles, with a very few giant cells in one of the fibrous lymph nodes. This seemed one of the most evident cases of healing of well advanced tubercles in the guinea pig that has been noted in this laboratory. The other animal is still alive and would have been killed for pathological examination, except that it was being used for blood studies.

(8) *Number 14 M:* Mono-ethyl P-nitraniline O-mercuric acetate, causes complete inhibition of growth of tubercle bacilli *in vitro*, in dilutions up to 1 in 100,000, and complete tuberculocidal action in dilutions of 1 in 1000 and 1 in 10,000 after twenty-four hours' exposure. The animals treated with this compound lived 100, 346, 422 and 692 days and two are still living at date of writing, after 816 days. They received 118, 399, 529 and 992 mgm. of the compound, while the two that are still living have received 1560 mgm. The first two to die showed generalized and fairly extensive tuberculosis of lymph nodes, spleen, liver and lungs. The third showed only the lymph nodes involved. The fourth showed some enlarged, hard, fibrous lymph nodes, apparently partly or wholly

healed, with spleen slightly enlarged and liver fatty, while the lungs showed a moderate bronchopneumonia. The two that are still living gave a negative tuberculin test by both methods of administration, skin and testis, and would probably exhibit much the same pathologic picture as the one described under *15 Na.*

Weight Curves: A classification of weight curves of tuberculous guinea pigs, treated and untreated was worked out by me (6). The average gains and losses in weights of the different sets of guinea pigs in the chronic tuberculosis of *series 3* were charted in curves which may be compared with that general classification. It may be stated that the controls, the *number 15 Na* set and the number *14 M* set belong in *II C* of

TABLE 2

Table of average increases and decreases of weights of chronic treated tuberculosis

NAME OF SET	DURATION OF DISEASE	MAXIMUM HEIGHT OF CURVE	TERMINAL HEIGHT	DURATION OF ASCENT	DURATION OF DESCENT	WIDTH OF SUMMIT	TYPE OF CURVE
	weeks	*grams*	*grams*	*weeks*	*weeks*	*weeks*	
Controls	100	620	200	62	28	55	II C
No. 26. M-Nitro dimethyl aniline P-mercuric acetate	82	310	135	31	20	51	II E
No. 27. O-nitro dimethyl aniline P-mercuric acetate	86	375	−100	59	27	30	II E
No. 29. P-nitro monomethyl aniline O-mercuric acetate	98	610	20	51	48	40	II E
P-nitroso phenol O-mercuric chloride	65	770	410	59	6	28	II D
No. 20. P-nitroso mercury Diphenyl	88	1050	590	55	33	32	II D
O-nitro phenol P-carbinol P-mercuric acetate	98	400	−70	67	35	60	II E
No. 15 Na 2, 2′ mercuri bis P-nitro phenol	111	690	280	60	51	35	II C
No. 14 M Mono ethyl P-nitro aniline O-mercuric acetate	114	660	380	78	34	46	II C

that classification, that set *number 20* and P-nitroso phenol O-mercuric chloride belong in *II D*, and *number 26, 27, 29* and nitro saligenin are more comparable to *II E*, in that the height of the summit is lower and the elevation much wider than most of the other curves. These therefore give the effect of a very broad flat elevation, while *II D* has a slow ascent with a sharp apex and rapid descent, and *II C* has a slow ascent and descent forming a broad elevation with rounded summit. Table 2 itemizes the average weight findings used in charting these curves.

CONCLUSIONS AND DISCUSSION

While these experiments scarcely permit us to draw a definite favorable conclusion regarding the therapeutic value of organic mercurial compounds in experimental tuberculosis in guinea pigs, some of the findings seem to indicate that in a slowly progressive chronic tuberculosis certain of the compounds do bring about a definite fibrosis and healing of tubercles in different organs, such as is not found in any of the control animals, however long they live. One of the most favorable-appearing compounds is a nitrophenol derivative, numbered *15* in our list, and another is a nitraniline derivative known as *14 M* in our list. The small doses used and hence the small amount found distributed in the organs may explain the lack of more definite results in these experiments. These compounds, in the doses used, have produced no nephritis in any animal examined, beyond a slight distention of tubules near the capsule and an occasional fibrosis of some glomeruli.

REFERENCES

(1) DEWITT: Jour. Infect. Dis., 1921, xxviii, 150.
(2) KHARASCH AND PICCARD: Jour. Amer. Chem. Soc., 1920, xlii, 1855.
KHARASCH AND JACOBSOHN: Ibid., 1921, xliii, 1895.
KHARASCH: Ibid., 1921, xliii, 1203.
KHARASCH AND CHALKLEY: Ibid., 1921, xliii, 607.
KHARASCH, LOMMEN AND JACOBSOHN: Ibid., 1922, xliv, 793.
(3) DEWITT: Jour. Infect. Dis., 1922, xxx, 363.
(4) ABELIN: Deutsch. med. Wchnschr., 1912, xxxviii, 1822.
(5) ELLIOTT: Jour. Amer. Med. Assoc., 1917, lxviii, 1693.
(6) DEWITT: Jour. Infect. Dis., 1920, xxvii, 503.

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THE INFLUENCE OF SILICA COMPOUNDS ON EXPERIMENTAL TUBERCULOSIS[1]

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. XXIX

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Silicates have been considered by a few clinicians and pharmacologists to exert a favorable influence in pulmonary tuberculosis, but on two entirely distinct grounds. One is based on the popular belief that persons who work at trades leading to the inhalation of certain silicious dusts are relatively free from pulmonary tuberculosis, presumably because the resulting pulmonary fibrosis helps to circumscribe and heal any existing tuberculous lesion (1). The same theory has been advanced concerning pulmonary anthracosis as a protective condition (2), and the reputed immunity of workers in lime dust has been attributed by some to a similar mechanical fibrosis, by others to hypercalcification of body fluids which is supposed to facilitate calcification and healing of tuberculous lesions. Landis (3) has reviewed the evidence on the effect of silicious dust on the lung, which seems to be conclusive that this is the form of pneumonokoniosis that leads to the greatest amount of injury to the pulmonary tissues, perhaps because of its gritty hardness. Coal dust seems to be best tolerated by the lungs, and plaster of Paris, clay and cement dust also produce much less injury than silica, quartz, flint, sandstone, emery and carborundum, as attested by the British Royal Commission on Metalliferous Mines and Quarries, and they lay particular emphasis on the harmfulness of fine crystalline silica as a cause of fibrosis.

This tendency to the production of pulmonary injury may, of course, be expected equally well to facilitate infection because of the tissue injury it produces, or to tend to healing by walling off tuberculous lesions. As a matter of evidence, however, this British Commission reported that there is "an excessive mortality from respiratory diseases,

[1] From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, University of Chicago.

and especially from phthisis," from inhalation of dust of quartz, quartzite, flint and sandstone. Also, the granite workers of Vermont are reported as having an excessive tuberculosis rate (Hoffman). The difficulty with this statement, however, lies in the fact that the chalicosis itself leads to conditions which clinically resemble fibroid phthisis, and hence it is not certain that the reputed high incidence of tuberculosis in stone workers actually exists. Landis states that in his own experience this mistake has often been made, and that, when pulmonary tuberculosis does develop in persons with chalicosis, it commonly runs a chronic course, as Rössle (4) found for porcelain workers. Landis found, however, an actual high tuberculosis death rate in potters, which he attributes to the dust acting as a carrier of tubercle bacilli. Such attempts as have been reported in medical literature to secure curative effects through inhalation of inorganic dust, by tuberculosis patients or by infected animals, seem to have been unsuccessful.

Of most value are the studies of Gardner (5) who caused guinea pigs to inhale Vermont granite dust and also tubercle bacilli of low virulence. The strain of bacilli used, when inhaled alone, produces pulmonary tubercles which heal completely after a short time, but in animals inhaling both dust and bacilli the lesions are more numerous, larger, with an equal amount of caseation but more fibrosis, and persisting much longer than in the animals that did not receive dust. Cesa-Bianchi (6) also reported that guinea pigs subjected to dust inhalation were more susceptible to tubercle bacilli injected subcutaneously, which produced chiefly pulmonary lesions in these animals, while in the controls the lesions were especially in the spleen and liver.

Gye, Purdy and Kettle (7) point out that the insolubility of silica cannot well account for its extensive fibroplastic effect, especially since the more insoluble carbon dust has much less effect. Having found that dissolved colloidal silica is a strong tissue poison, they suggest that inhaled silica is slowly converted into this toxic colloidal form, and by injuring the tissues reduces local resistance to tubercle bacilli. The obstruction to lymphatics by the proliferated tissue may also prevent removal of tubercle bacilli from the lung in a normal way.

Although Gardner's experiments fail to show any evidence of a beneficial effect from the pulmonary fibrosis induced by the dust, and even indicate some decrease in local resistance, it cannot be said that they demonstrate what the effect of an old fibrosis in man may be on a newly acquired infection, or on the localization of existing latent infections,

or on the progress of human phthisis which differs from pulmonary tuberculosis in the guinea pig in that the human subject has more immunological defense against the bacilli.

An entirely different influence of silica in tuberculosis has been described by several German investigators, inspired largely by the pharmacologist, Kobert. One of his pupils, Siegfried (8), has reviewed the literature on silica up to 1901, discussing its importance as the chief constitutent of the earth, its distribution in the vegetable kingdom, and its occurrence in the animal body. Despite its insolubility, small amounts of silica are absorbed, chiefly from plant foods, so that minute traces are found in all the tissues and in the circulating blood. Especially large amounts are said to occur in the hair and feathers, but here the question inevitably arises as to the possibility of mechanical contamination of the external appendages with the omnipresent silicates. Against this assumption, however, is the statement that the hair of newborn infants contains nearly as much silica as that of adults (0.083 to 0.103 per cent of the ash), and it is present in the hen's egg and in the feathers of the unhatched chick. Furthermore, it seems to be particularly abundant in the Wharton's jelly of the umbilical cord. This would also indicate that silica circulates in so diffusible a form that it can pass from the mother to the fetus, a point of importance in considering its possible therapeutic utilization.

Apparently, connective tissue is especially rich in silica, and some authors ascribe to this element an important function in determining the elasticity and tensile strength of fibrous tissue, an idea which may well be doubted in view of the very small amounts of silica that are present.

In this laboratory Broman (9) has failed to find that feeding silica to rabbits with healing severed tendons leads to an appreciable increase in strength of the new formed connective tissue. Analysis of normal rabbits' tendons indicated the presence of 0.167 per cent of dry weight as silica, which is much more than was found by us in the normal tissues of guinea pigs, namely, lymph nodes 0.069, lungs 0.0196, liver 0.0062 and spleen 0.011 per cent.

Of the viscera, especially large amounts have been described in the pancreas,[2] which some have looked upon as the chief depository of silica for the body needs, analogous to the iodine storage of the thyroid.

[2] Lüning (Die anorganisch Bestandteile des Pankreas, Dissert., Würzburg, 1899) found in the ash of 2 human pancreases the following: SiO_2, 0.93 per cent; Ca, 16.94 per cent; Mg, 0.37 per cent.

In 1901 Hugo Schulz (10) also took up the study of tissue silica, and himself confirmed the statement that connective tissue is comparatively rich in silica, beef tendons having about 0.48 per cent of SiO_2 in the ash and spleen capsule 0.45 per cent, as compared with muscle, 0.08 per cent. In man corresponding results were obtained as follows, in per cent of SiO_2 in ash: muscle, 0.0531; skin, 0.1484; tendons, 0.3385; dura mater, 0.3361; fascia, 0.2462. He comments on the fact that silica seems related to the supporting structures in all forms of life, and suggests that this element may have therapeutic value in diseases in which the connective tissues are concerned.

According to the same author (11), young tissues contain more silica than old; for instance, tendons in the aged contain 0.0408 gm. SiO_2 per kilo, in young persons, 0.0865; muscle, 0.0191 as against 0.0257; skin, 0.0385 as compared to 0.0510. The umbilical cord he found to contain about 0.24 gm. of SiO_2 per kilo of dry substance[3] most of which can be extracted with hot water, although its form of combination was not determined. He found from 0.033 to 0.0400 gm. SiO_2 excreted in the urine per litre, the largest amounts when the diet consisted chiefly of beans and bran bread.

Presumably, the chief source of silica in the animal economy is from vegetable foods which contain much silica; and it is an interesting fact that numerous popular herb teas contain considerable soluble silica, and the same is true of certain mineral waters, some of which have had a reputation as of value in the treatment of phthisis. Zickgraf (12) ascribes to the silica content of a certain water an improvement in the character of the leucocytes in tuberculosis as indicated by the Arneth formula.

Kahle (13) undertook, in the pathological laboratory of Rössle at Jena, an investigation of the effect on experimental tuberculosis of an organic silica preparation devised by Dr. Weyland of the same university, the chemical nature of which is not disclosed. He found that in 3 cases of phthisis the amount of SiO_2 in the urine was low (0.007 to 0.0093 gm. per day), but as he says nothing concerning the diet these

[3] It may be suggested that the high figure for the SiO_2 content of the umbilical cord, reckoned on the dry weight of the cord, may depend, at least partly, on the relatively high water content of the Wharton's jelly, since the SiO_2 content may vary with the total weight of the fresh tissues. If the chloride content, for example, were reckoned on the basis of dry weight of tissue, the umbilical cord would also seem to be particularly rich in chlorides.

figures have little significance. Similar low figures were obtained in 5 cases of cancer. However, the statement is made that the scars of healed pulmonary lesions showed no more SiO_2 than normal connective tissues, but stress is laid on the reputed presence of silica in healed calcified tubercles. We have recently investigated the silica content of healed calcified human tuberculous lesions (14), and have found that they contain only such silica as may have reached the tissues from external sources through inhalation of dust. Calcified peribronchial lymph nodes and pulmonary lesions contain a small proportion of silica, but not more than uncalcified peribronchial lymph nodes or lung tissues contain in adults. This silica is derived chiefly, if not entirely, from inhaled silicious dust, for calcified tuberculous mesenteric lymph nodes do not contain appreciable amounts of silica, and only a trace was found in a calcified tuberculous pleural exudate. Therefore, these analyses furnish no chemical evidence supporting the hypothesis that silica plays an important part in the healing of tuberculous lesions. Calcified tuberculous lesions resemble in composition other calcified pathological tissues and also normal bone.

Kahle infected guinea pigs with large doses of tubercle bacilli, and gave them 0.5 to 2 gm. daily of Weyland's preparation in their food. He states that after only a few days' treatment there occurred a marked increase in the fibroplastic reaction in the tubercles, leading after a time to the formation of dense fibrous tissue. These changes were observed in tubercles in any and all tissues, in the liver there being a veritable cirrhosis. These anatomical observations are corroborated by Rössle, who says that it was possible to distinguish the tissues of the treated animals from those of untreated controls by their anatomical characteristics without other information. Rössle also comments (4) on his own observations on the resistance to tuberculous infection of porcelain and cement workers with pneumonokoniosis, but apparently found no evidence of anything more than a mechanical action of inhaled insoluble particles and the resulting pulmonary fibrosis. He also points out the significance of Kahle's observation that the SiO_2 content of the pancreas is high in cancer and low in tuberculosis, as related to the reputed lack of coexistence of these two diseases in the same person.

In 1921 Kahle (15) made a more extended report. He agrees with Schulz that the normal human pancreas contains about 0.14 to 0.15 gm. SiO_2 per kg. dry weight, and in 20 cases of tuberculosis found figures

ranging from 0.01 gm. in caseous pneumonia to 0.28 gm. per kg. of dry pancreas in healed tuberculosis, but in active tuberculosis the figures were usually much below normal. In pregnant women low figures were found, supposedly because of transfer of SiO_2 to the fetus, and possibly accounting for the susceptibility of pregnant women to tuberculosis; in a case of puerperal miliary tuberculosis he found no SiO_2 at all in the pancreas. However, in some cases of active tuberculosis he found high figures for SiO_2 in the pancreas. The finding of Schulz, that in tuberculosis there is no decrease in the pancreatic silica, Kahle attributes to a failure to differentiate between active and healed tuberculosis. He reports one experiment with a dog, in which the production of large wounds led to a decrease in the SiO_2 excreted in the urine, attributed to retention in the new-formed connective tissue; after the pancreas was resected the urinary SiO_2 was greatly increased. This experiment was not confirmed by repetition. He describes 20 experiments in the silica treatment (Weyland preparation) of guinea pigs infected with large doses of human tubercle bacilli, which caused a marked fibroplastic reaction about the lesions, although none of the animals was cured.

Gye and Kettle (16), who found that intravenous injections of colloidal silica leads to fibrosis in the liver and kidney, investigated its therapeutic value in animals. Rabbits were inoculated intravenously with human tubercle bacilli, in doses which were just sufficient to cause lesions visible to the naked eye two months after injection. Some of the animals were treated by mouth, or by intravenous injection, with soluble silica and an equal number left as controls. "In no instance was the process of tubercularization delayed or prevented by the silica; in a certain number of animals the process (*sic*) seemed to be helped by the drug, but in general it had no material effect on generalized tuberculosis." Thoma (17), in a similar series of experiments, found evidence that the colloidal silicic acid protected rabbits against infection by human tuberculosis; but if the animals were already infected there was no beneficial effect, and, if anything, the disease was more extensive in the treated animals. Clinical experience with the same preparation was equally unsatisfactory.

Kühn (18), collaborating with Kobert's laboratory in Rostock, reports that his clinical experience has indicated to him that administration of silica for long periods is beneficial in tuberculosis and also is of prophylactic value. For this purpose he has used a vegetable tea rich

in silica, and also tablets containing colloidal SiO_2, which he believes is utilized, since an increased urinary excretion of SiO_2 was observed after their administration, and also an increase in the blood.[4] He believes that coincident silica and calcium treatment cannot be accomplished because insoluble compounds are produced, but he recommends alternation of the two at considerable intervals. While admitting the absence of analyses for silica of lungs with and without tuberculosis, like Kobert he assumes the vital importance of silica for the healing of tuberculous lungs. He reports (19) that "a series" of guinea pigs were given injections of colloidal silicic acid (amount not stated) and infected with tuberculosis. Half of the silica-treated guinea pigs died in "some weeks" of pneumonia and showed no trace of tuberculosis; the remainder were alive after four months showing no trace of tuberculosis, while the control animals had died after 4 to 6 weeks with widespread tuberculosis. "Similar results" were obtained in a second series. Unfortunately, the lack of details makes it impossible to estimate the value of these experiments, but Kühn does not hesitate to state that they show that "guinea pigs may be immunized against tuberculosis with silicic acid injections." He reports having treated over 400 cases of tuberculosis with silica in various forms (silicic acid injections, *Silicol* tablets, herb teas) and considers the procedure beneficial in that the course of the disease is made more chronic, but he is very vague and indefinite, admitting that "*sichere Beweise stehen noch aus.*" As far as we can find, Kühn has not reported the eventual outcome of his animal experiments, and so we do not know whether or not they confirm Kahle's work on the influence of silica on fibrosis.

Much analytical work on silica in tissues, secretions and foods has been done in Kobert's laboratory by Gonnermann (20), who gives figures for the SiO_2 content of many human tissues, but does not include the pancreas, so that no comparison can be made bearing on the supposed importance of this organ as a silica reservoir. Neither does he discuss the relative richness in SiO_2 of normal and tuberculous tissues. We may also mention the analyses of Breest (21), who found that the bodies of mice fed silicates contained a little more than controls,—up to 5 mgm. in the entire body. (A review and repetition of this work will be found in the experimental part of this paper.)

[4] A discussion of the theory of silica absorption is given by S. Loewe (Klin. Wchnschr., 1922, i, 278).

Kesseler (22), like Kühn, treated patients with a vegetable silicium (43 to 272 mgm. SiO_2 per day), as well as calcium and silica tablets, and found an increase in the number of leucocytes, although not so striking an improvement in the Arneth formula as some others have described. He believes that he has seen clinical improvement "although the disease did not seem to us, as it did to Kühn, to exhibit under this therapy a course quite other than usually seen." In a case so treated that came to autopsy, however, he observed a marked connective tissue formation and encapsulation of the numerous pulmonary lesions.

Helwig (23) says that administration of SiO_2 to either men or animals causes a temporary fall in the number of leucocytes followed by a rise, especially of the polymorphonuclear forms, which show an exceptionally active phagocytic power. The Arneth formula is much improved, and in Helwig's opinion there is much clinical improvement even in advanced ulcerative pulmonary tuberculosis. Unfortunately, he gives no adequate details of either animal experiments or clinical observations.

Hayek (24) makes the following statement concerning silica therapy: "Not very pleasing are the very uncritical reports concerning the brilliant results from the 'tea cures' that have come from the clinical side. I myself have never been able to observe these startling results, although I have applied the silicic acid therapy very frequently in chronic consumptives." He goes on to say that he has tried subcutaneous injections of colloidal silicic acid (v. Heyden) in patients suffering for a long time with repeated small hemorrhages, and that, although the results observed so far were not convincing, they were enough to encourage further investigation.

To recapitulate, according to the authors cited:

(1) Inhalation of silicious dust leads to more fibrosis and to more untoward clinical effects than is the case with other forms of dust. Statistical evidence indicates that rough silica dust inhalation leads to a high tuberculosis death rate, but it is not certain that this statistical result may not be due in considerable measure to erroneous inclusion of non-tuberculous dust-produced pulmonary disease in the reported phthisis deaths. On the other hand, there are numerous statements in the literature to the effect that workers in some silicious dusts, for example, cement workers, are protected from tuberculosis. Animal experiments suggest the production of a lowered resistance to pulmonary tuberculosis from dust inhalation, but are inconclusive. If there is any beneficial effect from inhaled silicious dust it probably depends on fibrosis from

mechanical irritation, and not on any chemical action that can be used as a basis for therapeutics.

(2) On the basis of a limited and inadequate amount of analytical evidence, it is believed that connective tissues contain more silica than other tissues. Fom this it is assumed that silica is an essential constituent of connective tissue, but with no proof whatever. It is also assumed that administration of silicic acid or its compounds by mouth will increase the amount of connective tissue formed in response to tuberculous infection, and in this way will facilitate healing of the lesions and restriction of the progress of the infection.

In support of this hypothesis are advanced the following items:

(a) Low silica content has been described in the pancreas in a very small number of cases of active tuberculosis.

(b) Subnormal quantities of SiO_2 have been found in the urine in a very few cases of tuberculosis, but without any consideration of the silica intake or fecal excretion.

(c) Silica has been found in a few calcified nodules in lungs; but it has also been absent from others, and the lungs always contain silica from inhalation. Calcified tuberculous lymph nodes in sites not reached by inhaled silica have been found practically free from silica.

(d) Some clinical benefit has been attributed to the use of silicic acid in pulmonary tuberculosis, but all the published reports lack even the slightest evidence of control, and at the best the results described are very slight and vague.

(e) A few experimental animals treated with silicic acid are said to have outlived the controls, but not sufficient details of the experiments are furnished to support this statement.

(f) Tuberculous guinea pigs treated with a silicic acid compound are said to have shown a much greater connective tissue reaction about the lesions than the control animals. The details of this work have not been reported in a way that it can be critically considered, and it has not yet been confirmed by independent investigation.

The only justification for giving any consideration to the influence of silica on tuberculosis lies in the large amount of literature and uncritical work that already exist. There is no evidence submitted in favor of silica therapy that will stand the slightest criticism. The only significant item is the reputed increased fibroplastic reaction in tuberculous guinea pigs treated with silica, attested by a competent pathologist,

and largely on the strength of this we have undertaken a reinvestigation of the influence of silica on experimental tuberculosis in the guinea pig.

EXPERIMENTAL

On June 14, 1922, 40 guinea pigs were injected subcutaneously with a salt solution emulsion of virulent human tubercle bacilli (Dr. DeWitt's culture, no. 4015). Twenty of these animals were given 0.5 mgm., moist weight, tubercle bacilli. Ten of these were fed 1 gm. of *Silicol*[5] daily, except Sunday, from the day of inoculation. Ten were not fed silicol.

Twenty guinea pigs were given a smaller quantity, 0.1 mgm. of tubercle bacilli. Ten of these were fed 1 gm. silicol daily in the same manner. Ten were not fed silicol. As controls, 11 normal guinea pigs were fed 1 gm. of silicol daily. The silicol was fed in 2 gelatin capsules, each containing 0.5 gm., and was entirely supplementary to the regular laboratory diet of hay, oats and carrots. The guinea pigs inoculated weighed from 295 to 520 gm. with an average of 355 gm. The normal controls were somewhat heavier, weighing from 375 to 607 gm., with an average weight of 504 gm.

The tuberculous infection was allowed to run its course in the inoculated guinea pigs, whether the animal was fed silicol or not, except in the following cases: 3 animals were killed because of paralysis of the hind legs due to large open ulcers (caused by secondary infection at the site of inoculation). Two animals were killed two months after the other animals in their respective sets had died. One died of unknown cause seven days after injection; this animal is not included in the experiment.

As control material for chemical analysis and microscopic study, one normal silicol-fed guinea pig was killed for every 4 of the tuberculous animals.

At autopsy the lymph nodes, spleens, liver and lungs, all of which exhibited tuberculous lesions in the majority of cases, were removed, freed of excess blood by wiping and placed in 95 per cent alcohol until analyzed. Small portions of all tissues showing tuberculous involvement were saved for histological study. The ten livers of the animals of each series were analyzed together, likewise the ten lungs, spleens and the lymph nodes (intratracheal, parapan-

[5] This is described by the manufacturer (E. Laves, Lecinwerk, Hannover) as a SiO_2—casein—metaphosphate, containing about 25 per cent of SiO_2 in colloidal form. This preparation was selected as the one used by Kühn in some of his experiments. According to Zuckmayer, the silica administered in this preparation is absorbed in considerable amounts, and slowly excreted through the urine. An analysis of the preparation used in our experiments gave 26 per cent of SiO_2. The material is furnished in the form of a dry powder, readily administered in capsules. Theoretically, it is not broken up or absorbed until it reaches the intestines, thus avoiding the possibility of gastric irritation which is troublesome with some simpler silica compounds. We observed no harmful effects from feeding these comparatively enormous doses of silicol to guinea pigs.

creatic, inguinal and iliac) of each series were collected and analyzed. The tissues were removed from the alcohol, cut into small sections and dried at 110°. The liver tissue was extracted with ether for six hours in a soxhlet to avoid any error due to varying quantities of fat. The tissues were then ashed in new nickel crucibles, and the silica determined by the method of Lenher and Truog (25) with slight modifications adapted to smaller quantities of silica. The ash was removed to a platinum dish by means of a platinum spatula and dilute HCl. The mixture was evaporated to dryness on the steam bath. The ash was treated with 30 cc. of HCl (sp. gr. 1.07), and the mixture was evaporated to dryness. The residue was treated with HCl (sp. gr. 1.10), covered and heated on the steam bath for ten minutes. The solution was diluted with 5 cc. H_2O and filtered immediately. This filtration removes most of the silica. The filtrate was evaporated to dryness and dehydrated for two hours at 110°. This residue was taken up in 8 cc. HCl (sp. gr. 1.10), covered and heated on the steam bath for 5 minutes, diluted with 25 cc. H_2O, and filtered immediately. The dehydration was repeated if the amount of SiO_2 present on the last dehydration warranted the procedure. The two filter papers containing the SiO_2 were placed in a platinum crucible and ignited, ashed and weighed. The silica was determined by loss on volatilizing with hydrofluoric acid, after the addition of a few drops of dilute sulphuric acid. The amount of silica determined in the reagents used never exceeded 0.2 mgm. Control analyses of a pure SiO_2 preparation showed that the method was accurate for 0.5 mgm.

To check the results of the first experiment, on October 18, 1922, another series of 40 guinea pigs was inoculated in the same manner, 20 animals receiving the same larger injection and 20 animals receiving the smaller quantity of tubercle bacilli. Ten of each series were fed only 0.5 gm. silicol daily, instead of 1 gm. as in the first experiment. The inoculated animals weighed from 340 to 500 gm., with an average weight of 393 gm. The normal controls weighed from 360 to 490 gm., averaging 404 gm. There was no evidence of beneficial effect produced by the silicol treatment either in greater length of life of the treated animals or in the extent of the tuberculous involvement seen at autopsy. The silicol-fed pigs which had received the larger quantity of tubercle bacilli lived, on an average, 88 days, while their untreated controls lived on an average, 108 days. The silicol-fed animals which had received the smaller injection of tubercle bacilli lived, on an average, 124 days, while their untreated controls lived the same length of time. The amount and extent of tuberculosis grossly visible at autopsy was the same, as nearly as could be estimated, in treated and untreated animals.

ANATOMICAL STUDY

The degree of tuberculous involvement was estimated in the following manner: The four chief sites of tuberculosis were selected (lungs, spleen, liver and lymph nodes) and the extent of tuberculosis in each graded as follows: slight, 1; moderate, 2; marked, 3; extreme, 4. Hence, if an animal showed severe tuberculosis in all these four tissues it would be graded 16, the maximum. These gradings were added and averaged for the 40 that received silicol and the 39 that did not, the figures being 8.45 for the treated and 8.58 for the untreated. This difference is certainly within the limit of error for a method of such a limited exactness, and corroborates the impression gained by observation of the autopsied animals that there was no constant difference between the two sets of animals.

It was thought that the 1 gm. dose of silicol daily might be large enough to be toxic or irritating although no definite evidence of this was observed in the animals. A section of the small intestine of a normal silicol-fed guinea pig was studied microscopically, but no changes were found.

The influence of silica on the histological structure of the tuberculous lesion, and especially the amount of fibroplastic reaction, was determined in the following way: Sections were taken from the liver, spleen, lungs and lymph nodes of every animal used in these experiments, whether tuberculous, normal, silica-fed or not. Each section was then examined microscopically, with no knowledge on the part of the examiner as to whether the animal had received silicol, or how long it had survived infection. The grade of fibrosis in reaction to tuberculous infection was then estimated and recorded, graded for each organ as from 0 to 4+. After this had been done for all the tissues in all the animals, the total degree of fibroplastic reaction for each animal was estimated by considering the amount of involvement and amount of fibrosis, each animal being graded according to the following standard:

0—Little or no fibroplastic reaction, the tuberculous lesions everywhere showing merely necrosis with little or no fibrous tissue about them.

1+—Slight fibrous tissue formation about nearly all or all the lesions; this is generally most marked, if present, in the hepatic lesions, least in the spleen.

2+—Moderate fibrous reaction about all lesions, or slight about some and marked about others.

3+—Marked fibrous reaction about all lesions.

After this had been done in 71 animals, in which there were sufficiently extensively tuberculous lesions to make the results of value, the gradings were compared with the amount of silicol that had been fed. It was found that the *amount of fibrosis thus objectively determined bore no relation whatever to the amount of silicol fed*, there being exactly as much fibrosis in the animals that received no silicol whatever. This is shown in the following summary:

GRADE OF FIBROSIS	NUMBER OF ANIMALS	NOT FED SILICOL	FED SILICOL
3	8	4	4
2	12	6	6
1	31	15	16
0	20	10	10
Total.....	71	35	36

It is seen that almost exactly the same number of silicol-fed and control animals exhibit each grade of fibrosis. Furthermore, in the animals fed silicol no difference exists in respect to fibrosis, whether the animals were given 0.5 or 1.0 gm. doses of silicol. It was also found that the amount of fibrosis was not determined in these animals by the size of the dose of tubercle bacilli with which the animals were injected.

These constant findings, we believe, completely refute the statement that the administration of silica to tuberculous guinea pigs increases the amount or character of fibrous tissue reaction. This varies greatly in different animals, why we do not know, but certainly it does not depend on the feeding of silicol.

CHEMICAL STUDY

Silica analyses were made of the lungs, lymph nodes, spleens and livers of normal guinea pigs. In order to have comparable weights of tissue for analysis, 31 spleens and the lymph nodes from as many animals were collected and analyzed. The lungs and livers were obtained from 10 normal guinea pigs. The tissues of each kind were united and analyzed as single lots, in order to secure enough silica to have figures of value (two or more milligrams). The results of these analyses are given in table 1, which shows that the lungs and lymph nodes contain much more silica than the liver and spleen. This may well be because each receives deposits of silicious dusts from outside the body.

The analytical figures for the silica content of the organs of normal guinea pigs fed silicol are shown in table 2. These indicate that silica feeding of normal animals leads to a definite increase in the amount of silica present in the liver and spleen, but not in the lungs. Inconstant results were obtained for the lymph nodes, presumably since when peribronchial nodes are included there are varying amounts of inhaled silicious dust present. Possibly the mesenteric nodes may contain more or less silicate absorbed directly from the intestines of animals fed large amounts of silica preparations.

TABLE 1

Silica content of organs of normal guinea pigs

	MGM. SiO_2 IN 100 GRAMS DRIED WEIGHT			
	Lungs	Lymph nodes	Livers	Spleens
	19.6	60.2	6.2	10.9

TABLE 2

Silica content of organs of normal guinea pigs fed silicol

NUMBER OF ANIMALS		AMOUNT SILICOL FED DAILY	AVERAGE NUMBER OF DAYS FED				
11		1 gm.	137	22.3	59.6	13.6	24.5
10		0.5 gm.	84	16.0	124.1	24.1	35.6
Average				19.15	91.8	18.8	30.05

TABLE 3

Silica content of organs of tuberculous guinea pigs fed silicol

	AMOUNT BACILLI INJECTED						
10	0.1 mgm.	1 gm.	124	20.5	118.7	0.2?	20.0
10	0.1 mgm.	0.5 gm.	71	13.7	54.3	11.3	5.3
10	0.5 mgm.	1 gm.	88	10.7	60.0	11.3	14.3
10	0.5 mgm.	0.5 gm.	63	8.0	35.0	8.2	4.5
Average				13.2	67.0		11.02

TABLE 4

Silica content of organs of tuberculous guinea pigs not fed silicol

10	0.1 mgm.		124	14.5	30.3	27.6	20.5
10	0.1 mgm.		87		25.4	21.9	12.2
9	0.5 mgm.		117	14.1	37.2	20.9	13.7
10	0.5 mgm.		87	18.3	47.5	14.1	4.5
Average				15.6	33.8	21.1	12.7

On the other hand, tables 3 and 4 show that feeding silicol to tuberculous pigs caused no increase in the quantity of silica present per 100 gm. dried weight of liver, spleen and lung. It is to be noted that the tuberculous organs of silica-fed guinea pigs show less silica than corresponding

organs in normal guinea pigs fed silica. This would suggest that such silica as is present is in the normal tissue, and the added cells and exudate of the tuberculous lesions, reduce the proportion of silica, since we find that the liver, lungs and spleen of tuberculous animals not fed silicol commonly contain as much or more silica than the tissues of tuberculous animals that were fed silicol. The occasional high figures in the lymph nodes are most probably explained by inhalation of excessive amounts of silica dust present in the cages of animals fed silicol. We found similar occasional excessive figures for calcium in the lymph nodes of animals fed calcium salts (14).

That normal peribronchial lymph nodes do ordinarily contain more silica than other lymph nodes is shown by the following observation: The lymph nodes from over 100 nontuberculous guinea pigs of various weights, killed in the laboratory in other experiments, were separated into two groups, (1) the peribronchial nodes and (2) all other nodes (mesenteric, inguinal and iliac), and the two lots were analyzed for silica. The results were:

	Per 100 gm. dried weight
Peribronchial lymph nodes	45 mgm. SiO_2
Other lymph nodes	28 mgm. SiO_2

A similar large series of actively tuberculous animals, not fed silicol, which had been killed in the course of another experiment in our laboratory, were studied in the same way with the following result:

	Per 100 gm. dried weight
Peribronchial lymph nodes	24.1 mgm. SiO_2
Other lymph nodes	26.4 mgm. SiO_2

If we compare these figures with those for the lymph nodes in tables 1 to 4 we find evidence that tuberculosis of the lymph nodes reduces the proportion of silica in the node, and that the excessively high figures for the lymph nodes in some of the animals fed silica probably depend on inhalation of silica dust from the fecal material in the cages. As no separation of the peribronchial from the other lymph nodes was made in the silicol-fed animals, it cannot be said definitely that the great increase in SiO_2 in the nodes of some groups of these guinea pigs was due to inhalation. Presumably the low silica figure for the lymph nodes of untreated tuberculous guinea pigs depends on the great increment of new tissue elements and exudate lowering the proportion of silica present in the original tissue.

Since the organs analyzed represented many different stages of tuberculous infection, an experiment was planned to determine whether the silica content varied with the progress of the disease. Spleens were classified as moderately tuberculous when they showed discrete active tubercles separated by normal tissue. The severely tuberculous spleens were almost entirely necrotic and usually very much enlarged, showing little. if any, normal tissue macroscopically. The results follow:

	Per 100 gm. dried tissue
Moderately tuberculous spleens	15.7 mgm.
Severely tuberculous spleens	9.0 mgm.

These figures support the assumption made previously that the more tuberculous change in a tissue the smaller the proportion of silica.

The pancreatic tissue from 41 tuberculous guinea pigs was analyzed for silica and also the pancreatic tissue from 31 normal guinea pigs, no silica having been fed in either group, with the following results:

	Per 100 gm. dried tissue, fat free
Normal pancreatic tissue of tuberculous guinea pigs	27.8 mgm.
Pancreatic tissue of normal guinea pigs	16.7 mgm.

The higher figure for the nontuberculous pancreas of actively tuberculous guinea pigs does not harmonize with the hypothesis of Kahle that the pancreas is a storehouse for silica which is depleted in active tuberculosis.

ABSORPTION OF SILICA BY MICE

In the introductory part of this paper (p. 324) reference was made to the work of Breest (21) whose experiment was performed as follows:

Nine mice, three months old, from two related strains, were fed 75 per cent wheat flour and sweetened condensed milk. After feeding 15 days, there was one day fast. He fed 75 mgm. SiO_2 in 15 daily 5 mgm. doses to one group, and to the other, 15 daily 6 milligram doses. Three were given colloidal silica hydrate, 3 sodium silicate, 2 neutralized silicate and only one was used as a control. Apparently, he analyzed the entire mice, hair, intestines and all. The control mouse had 0.15 per cent SiO_2 in its total ash, this being a total of 0.5 mgm. The other mice had from 0.19 to 1.22 per cent SiO_2. The highest figures were given by the mice fed the neutralized silicate. As these figures amounted to but 4.5 mgm. total silica retained, they represent but a very small proportion of the ingested silica. Actually, there was no appreciable increase in two of the mice, which had less than 1 mgm. SiO_2 in the ash. Nevertheless, Breest concluded that all these mice had absorbed and retained silica.

However, he also analyzed two untreated mice from another dealer and found in one 0.66 and in the other 2.72 per cent. He discussed, but did not explain the wide variations, including his own normal mouse with 0.15 per cent. He considered that, in view of his low figures with colloidal silicic acid, this was not absorbed. He gave no consideration to inhaled silica or silica in fur and intestine as a source of error. As a matter of fact, his figures do not at all establish his conclusion that silica had been absorbed, for this is based on comparison with a single mouse that gave a particularly low figure. Had he used as a basis the much higher figures which he found in two other normal mice the necessary conclusion would have been that feeding silica depletes the body silica. As a matter of fact the lack of adequate control makes the results valueless.

TABLE 5

GROUP	SKINNED BODIES OF THE MICE			SKIN, TAIL AND ALIMENTARY CANAL		
	Fresh weight (3 mice)	Weight of SiO_2 found	Per cent SiO_2	Fresh weight (3 mice)	Weight of SiO_2 found	Per cent SiO_2
Mice not fed silicol						
1	31.6736	0.0004	0.0012	12.9185	0.0025	0.019
2	29.0074	0.0005	0.0016	14.4165	0.0033	0.022
3	29.2720	0.0008	0.0027	13.0692	0.0009	0.0068
Mice fed silicol						
1	32.0713	0.0008	0.002	15.7484	0.0067	0.042
2	32.9484	0.0006	0.001	15.4012	0.0086	0.055

In order to study the question more fully the following experiment was performed:

Twelve mice were fed a diet consisting of two parts coarse yellow corn meal and one part whole milk powder (Dryco), to which had been added filter paper cut into small pieces for roughage. Twelve other mice were given the same diet, to which had been added 10 per cent silicol. One small carrot was given to each set daily.

At the end of twelve days 9 of the mice not fed the food with silicol were alive while 7 of those fed silicol lived. All food was removed from the cages on the evening of the twelfth day, and the mice were killed with illuminating gas the following morning. Their skins, tails and alimentary canals were collected and weighed separately, and then analyzed in groups of 3 each. The remaining skinned bodies, with the alimentary canal removed, were also weighed separately, and then analyzed in groups of 3, to determine how much silica had entered and been stored in the body as a result of silicol feeding. The results are given in table 5.

From these figures it is seen that feeding of silicol has not increased the amount of silica absorbed into the bodies of our 6 mice, the total amount in all being at the minimum quantity determinable even when three entire mice are used for each analysis. The skin and alimentary canal of normal mice contain much more silica than all the rest of the body. In animals fed silicol the skin and alimentary canal, removed twelve hours after feeding was stopped, contain more than twice as much as in the controls, probably part being in dust adherent to the hair and part silicol not yet eliminated from the intestines.

SUMMARY

A review of the literature on the use of silica preparations in the treatment of tuberculosis, human and experimental, leads to the conclusion that the evidence so far presented is far from establishing that beneficial results are obtained from the use of this element. On account of the large amount of conflicting or inadequately controlled testimony in respect to the effect of silica preparations on experimental tuberculosis, a reinvestigation of this subject has been carried out. It was found that administration of an organic silica preparation (*Silicol*, that is, SiO_2-casein-metaphosphate) to tuberculous guinea pigs does not increase the duration of life or modify the extent of tuberculous involvement of the tissues. Microscopic study showed absolutely no difference in the degree of fibroplastic reaction to tuberculous infection of the lungs, livers, spleens and lymph nodes in guinea pigs fed this silica preparation as compared with similarly infected animals not given silica treatment. Chemical examination of the entire bodies of mice fed silicol for twelve days showed no appreciable increase in the silica content in the entire body, from which the skin and intestine had been removed as contaminated with adherent but unabsorbed silica. Silicol feeding to normal guinea pigs led to an apparent increase in the amount of silica in the liver and spleen, and sometimes in the lymph nodes. Feeding of silicol to tuberculous guinea pigs caused no increase in the proportion of silica present in the tuberculous livers, spleens and lungs as compared with corresponding tuberculous organs in animals that were not fed silicol. Extensive tuberculous change in organs reduces the silica content below normal, whether the animals are fed silica or not, presumably because the exudative and new tissue elements reduce the proportion of silica. The pancreatic tissue of

tuberculous pigs contains more silica than the pancreas of nontuberculous guinea pigs, a finding which is not in harmony with the theory that the pancreas is a storehouse for silica which is depleted in active tuberculosis. The silica content of the lymph nodes of guinea pigs fed silicol is sometimes much higher than control animals, whether tuberculous or not, but usually there is no difference. These irregular figures probably depend on inhalation of silicious dust.

REFERENCES

(1) See VOLLRATH: Beitr. z. Klin. d. Tuberk., 1921, xlvii, 237.
(2) See HAYTHORN: Jour. Med. Res., 1913, xxix, 259.
(3) LANDIS: Jour. Indust. Hyg., 1919, i, 117.
(4) RÖSSLE: Beitr. z. Klin. d. Tuberk., 1921, xlvii, 325.
(5) GARDNER: Amer. Rev. Tuberc., 1920, iv, 609.
(6) CESA-BIANCHI: Ztschr. f. Hyg., 1913, lxxiii, 166.
(7) GYE, PURDY AND KETTLE: Brit. Jour. Exp. Path., 1922. iii, 75, 86 and 241.
(8) SIEGFRIED: Arch. de Pharmacodynamie, 1901, ix, 225.
(9) BROMAN, JESSIE R.: Amer. Rev. Tuberc., 1923, viii, 337.
(10) SCHULZ: Pflügers Arch. ges. Physiol., 1901, lxxxiv, 67; München. med. Wchnschr., 1902, xlix, 440.
(11) SCHULZ: Pflügers Arch. ges. Physiol., 1902, lxxxix, 112.
(12) ZICKGRAF: Ztschr. f. inn. Med., 1908, xxix, 509.
(13) KAHLE: München. med. Wchnschr., 1914, lxi, 752.
(14) MAVER AND WELLS: Amer. Rev. Tuberc., 1922, vi, 649.
(15) KAHLE: Beitr. z. Klin. d. Tuberk., 1921, xlvii, 296.
(16) GYE AND KETTLE: Lancet, 1922, cciii, 855.
(17) THOMA: Deutsche Arch. klin. Med., 1922, cxli, 175.
(18) KÜHN: Therap. Monatschi., 1919, xxxiii, 201; München. med. Wchnschr., 1920, lxvii, 253.
(19) KÜHN: Ztschr. f. Tuberk., 1920, xxii, 320.
(20) GONNERMANN: Ztschr. physiol. Chem., 1917, xcix, 255; Biochem. Ztschr., 1918, lxxxviii, 401; 1919, xciv, 113; 1919, xcv, 286.
(21) BREEST: Biochem. Zeitschr., 1920, cviii, 309.
(22) KESSELER: Deutsche med. Wchnschr., 1920, xlvi, 239.
(23) HELWIG: Med. Klin., 1921, xvii, 1266.
(24) HAYEK: Das Tuberculose-Problem, 2nd Ed., 1921, p. 232.
(25) LENHER AND TRUOG: Jour. Amer. Chem. Soc., 1916, xxxviii, 1050.

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THE INFLUENCE OF SILICA ON THE STRENGTH OF NEWLY FORMED CONNECTIVE TISSUE[1]

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. XXX

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Certain observers (1) having found that connective tissues contain somewhat more silica than other tissues, the hypothesis has been advanced that silica is an important factor in the tensile strength of connective tissue (2). Although the actual amount of silica present in connective tissue is so very small that this assumption may well be doubted, nevertheless it has served as the basis of a suggested method for the treatment of tuberculosis. Various silica-containing teas or drugs are being given with the hope of increasing the silica content of the tissues, and with the further hope that this will increase the amount and improve the quality of the connective tissue which forms the defensive reaction about the tuberculous lesions.[2] In order to learn whether the administration of silica actually does modify the strength of new formed connective tissues, a series of experiments was undertaken, following certain suggestions and technique found in an article by Lewis and Newcomer (3).

Their method was based upon a very early article by Paget (4). The object of their work was to study quantitatively the connective tissue factor in wound-healing. They found, however, that there was a wide variation in the tensile strength of connective tissue formed in response to surgical injury, and that there was no demonstrable relationship between the connective tissue repair following a routine surgical operation and natural resistance to inoculation tuberculosis in rabbits. They suggested the use of their method in the study of certain factors

[1] From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, University of Chicago.

[2] An investigation of the influence of silica on experimental tuberculosis is published from this laboratory by Maver and Wells in the preceding article, AMERICAN REVIEW OF TUBERCULOSIS, 1923, viii, 318.

which might affect wound-healing, either through local or systemic application.

The method devised by Paget consisted in suspending weights to the Achilles tendon of a rabbit some days after operative division in order to learn the amount of tensile strength the new formed tissue possessed. Lewis and Newcomer carried out virtually the same procedure, using as the test weight a pail into which sand was run until the tendon broke.[3] Examination of the figures given by these authors leaves much question as to the quantitative value of the procedure, for great variations are found, ranging in one series of untreated animals from 4120 to 10600 gm., in another series from 4920 to 13750 gm. Therefore, the mere fact, that the averages of a large number of animals in two series are approximately the same, would seem to have little significance. Nevertheless, for lack of a better method, and particularly to test out this method, it was adopted with certain modifications to determine what effect, if any, the systemic administration of silica might have upon the healing of severed Achilles tendons.

PROCEDURE

The animals chosen were between one and two kilos in weight and of as near the same age as possible. They were fed once daily for five days two gram capsules of Silicol.[4] On the fifth day they were operated upon under light ether anesthesia. The region over the Achilles tendon was washed with green soap, shaved, and cleaned with alcohol. The tendon on both sides was severed cross-wise through a small longitudinal slit in the sheath. The tendon sheath and skin were closed with a single catgut stitch. Neither hemorrhage nor infection resulted. After the operation the silicol medication was continued for ten days more. The rabbit was then killed, the tendons were dissected free from the other structures and their tensile strength tested by a device similar to that used in testing the tensile strength of commercial catgut sutures, this being found to be more satisfactory than the weight method used by Lewis. One end of the tendon was fastened to a spring scale, the other, by means of copper wire, to a small drum upon which it could be slowly and steadily wound up until the tension on the tendon caused it to break. The reading on the scale was made by means of a stop gauge. In a few instances the tendon pulled out of the muscle without breaking. Such instances are

[3] A similar method has been used in a study of the strength of healing, sutured nerves by Miller (*Archives of Surgery*, 1921, lxxxviii, 167).

[4] Silicol is a commercial preparation described as SiO_2—casein—metaphosphate (E. Laves, Lecinwerk, Hannover) containing about 25 per cent of silica. It has been shown that silica is absorbed from the intestine when this preparation is fed.

recorded, however, because this material formed a part of the chemical analysis. The results are given in table 1.

TABLE 1

WEIGHT OF RABBIT AT BEGINNING OF EXPERIMENT	TENSILE STRENGTH OF RIGHT TENDO ACHILLES	TENSILE STRENGTH OF LEFT TENDO ACHILLES
grams		
1360	Broke at muscle margin	8744 gm.
1450	8541 gm.	7823 gm.
1530	6338 gm.	Broke at muscle margin
1670	Broke at muscle margin	Broke at muscle margin
1385	6328 gm.	7003 gm.
1350	7698 gm.	6638 gm.
2310	9300 gm.	6675 gm.
1040	7250 gm.	10120 gm.
2150	9875 gm.	7540 gm.
1920	8590 gm.	6720 gm.
1350	7600 gm.	2940 gm.
1644	10460 gm.	8522 gm.
Average 1596	8198 gm.	7272 gm.

It will be noted that there was a rather wide discrepancy between the tendons of the same rabbit, although the average strength of the two series differs by only a little over 10 per cent. Evidently these averages mean but little when there are such great variations between the two tendons of the same individual.

In order to control the results a series of normal animals, which had not been fed silicol, were operated upon and their tendons tested in an identical manner. The results were again very variable and are given in table 2.

TABLE 2

WEIGHT OF RABBIT AT BEGINNING OF EXPERIMENT	TENSILE STRENGTH OF RIGHT TENDO ACHILLES	TENSILE STRENGTH OF LEFT TENDO ACHILLES
grams		
1500	Broke at muscle margin	9810 gm.
1250	Broke at muscle margin	Broke at muscle margin
1580	9500 gm.	7850 gm.
2159	9545 gm.	12074 gm.
1818	10900 gm.	10450 gm.
2040	11800 gm.	11250 gm.
1800	10980 gm.	12150 gm.
Average 1735	10545 gm.	10594 gm.

Here the averages are close enough, but there are still wide variations between the two tendons from the same animal. The figures do show,

however, that repair of the tendons had not in ten days restored them to normal strength, for in three pairs of control rabbits the unoperated tendons did not break at all, the muscle separating from the tendon in long strands.

Chemical analyses were made by Miss Mary E. Maver of all tendon material to determine the percentage of silica present in normal rabbit tendons, that present in tendons ten days after operation in rabbits which had been given silicol, and in those which had not had silicol.

28 normal rabbit tendons yielded 0.167 per cent dry weight of silica.

24 operated, silicol-fed rabbits, 0.109 per cent dry weight of silica.

14 operated tendons, without silicol feeding, 0.079 per cent dry weight of silica.

Although the average tensile strength of the tendons from rabbits which had not been fed silicol was greater than that of those silicol-fed the percentage of silica found was less. In other words, the tendons which chemically contained the greater amount of silica had less tensile strength, and the tendons which had been operated upon, in both the silicol-fed and the non-silicol-fed, contained less silica than the normal tissue.

SUMMARY

No satisfactory conclusions can be drawn from these experiments, owing to the very wide natural variation in the tensile strength of healing, severed Achilles tendons. There was no correlation between the two tendons of the same animal, nor between the individuals of the series. Too many factors may be recognized as having a bearing upon the tensile strength of healing, severed tendons, to permit of any interpretation of the effect of medication upon those which are undergoing repair after surgical procedure, however painstakingly that procedure may be conducted. At least, it may be said that no direct relation was found to exist between silica content and tensile strength, and no evidence was obtained that silica administration in any way strengthened the healing tendon.

REFERENCES

(1) Schulz: Pflügers Arch., 1901, lxxxiv, 67; München. med. Wchnschr., 1901, xlix, 440.

(2) Siegfried: Review. Arch. de Pharmacodyn, 1901, ix, 225; more recent literature is given by Wells, DeWitt and Long, Chemistry of tuberculosis, 1923, p. 426.

(3) Lewis, P. A., and Newcomer, H. S.: The functional value of newly formed connective tissue, Jour. Exp. Med., 1919, xxix, 369.

(4) Paget, J.: Lectures on surgical pathology, London, 1853, i, 271.

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HEMATOLOGICAL STUDIES ON EXPERIMENTAL TUBERCULOSIS OF THE GUINEA PIG[1]

II. THE EFFECT OF CERTAIN DRUGS ON THE BLOOD PICTURE IN TUBERCULOSIS

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. XXXI.

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In the first paper of this series (1) we studied the blood picture in nontreated tuberculosis of the guinea pig and concluded in part that:

1. Normal guinea pigs have a very uniform blood picture, particularly characterized by a seasonal variation of the hemoglobin content of the red cells, a high lymphocyte count, and a high eosinophile count coincident with the appearance of Kurloff bodies.

2. An acute infection with tuberculosis produces a characteristic blood picture, in which there is a rapid loss in erythrocytes with a relatively greater decrease in hemoglobin, a terminal leucocytosis or leucopenia, a progressive decrease in lymphocytes, a moderate preterminal increase in endothelial cells, and an absence of eosinophiles.

3. Chronic tuberculosis produces a blood picture quite different from that of acute tuberculosis. It is characterized by a fluctuation in all elements, a relatively low hemoglobin content of the erythrocytes which is not progressive but shows the normal seasonal variations, and absence of any reaction of the lymphocytes, and an eosinophilia which cannot, however, be distinguished from that associated with Kurloff bodies.

The original paper gives in full the methods and technique used, charts and tables of results, and discussions of the Kurloff bodies. The same methods have been now applied to the treated animals. The treatments employed have all been chemotherapeutic. For the chemistry of these drugs, their bactericidal powers, methods of treatment and effects on the

[1] From the Department of Pathology, University of Chicago, and the Otho S. A. Sprague Memorial Institute.

tuberculous animals, reference should be made to other papers by DeWitt (2) (3). It is the purpose of this paper to study only the blood pictures of the animals that were being treated, and to determine whether the drugs in any way affected the usual changes in the blood picture that have been observed to occur in tuberculosis.

The animals which were examined had been treated with the following drugs:

1. No. 29. Paranitro-orthoacetoxymercuri-monomethylaniline.
2. Methylene blue.
3. Mercuric chaulmoogric acid.
4. No. 44A. Mercurized methylene blue.
5. New methylene blue GG.
6. Neutral red.
7. No. 56. 4-hydroxy-3-5-diacetoxymercuri-trimethylphenylammonium acetate.
8. No. 27 L.C. Mercuri-bis-3-3'hydroxy-5-5'trimethylphenylammoniumacetate.

Nontreated tuberculous animals were examined with each set as controls, and in some cases nontuberculous treated animals were also examined. The routine blood examination was made on each animal usually once in two weeks throughout the period of their infection and treatment.

1: No. 29. Paranitro-orthoacetoxymercuri-monomethylaniline: Four guinea pigs which were being treated with this drug were examined, as well as 4 nontreated tuberculous controls and 5 nontreated, nontuberculous controls. The infection was of the chronic type, all of the animals living for at least one year after the inoculation, and the blood picture of the nontreated controls showed the typical blood changes of chronic tuberculosis. Typical results in these animals are given in chart 1. The results of the blood examinations of the nontreated guinea pigs, both the tuberculous and the nontuberculous, were reported in the first paper of this series.

The important difference in the blood picture of the treated and the nontreated animals was that the usual eosinophilia did not appear in the treated guinea pigs. As all of the animals had Kurloff bodies, there was an eosinophilia in the nontuberculous controls as well as in the tuberculous controls. Thus, in 4 nontreated tuberculous guinea pigs the average eosinophile count for one year (59 examinations) was 140 per cmm. of blood with 460 per cmm. as the maximum figure. In 4 treated tuberculous animals the average for the same period (56 examinations)

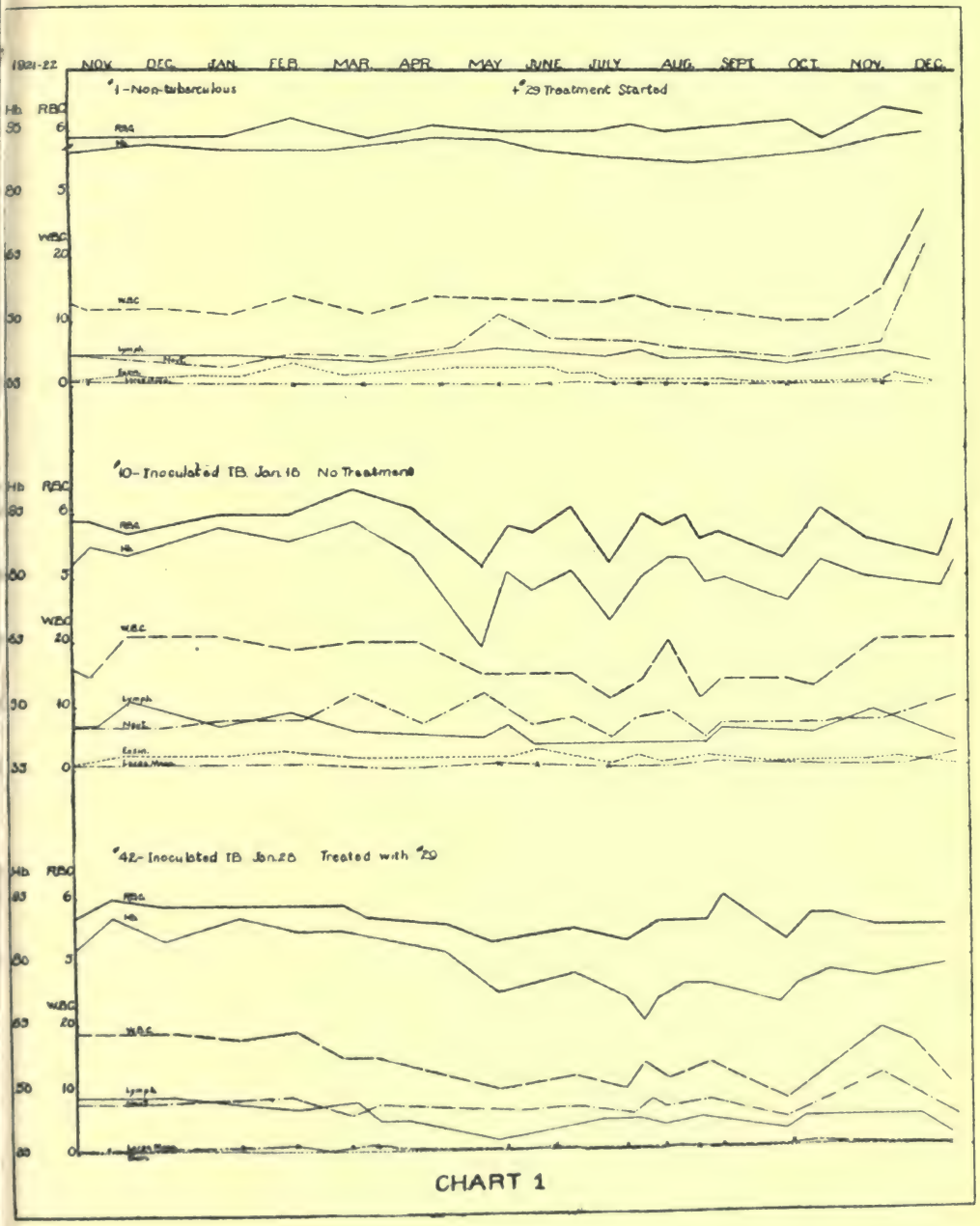

1921-22 NOV. DEC. JAN. FEB. MAR. APR. MAY JUNE JULY AUG. SEPT. OCT. NOV. DEC.
#1 - Non-tuberculous
+#29 Treatment Started
Hb RBC WBC
#10 - Inoculated TB Jan. 18 No Treatment
#42 - Inoculated TB Jan. 28 Treated with #29

CHART 1

was 25 per cmm. with 80 per cmm. maximum. Thus the eosinophile count of the treated animals was only 16.4 per cent of the nontreated.

In five nontuberculous animals the average eosinophile count for the first six months of the same period was 300 cells per cmm. of blood. For the last six months 3 of these animals were treated with no. 29 in the same doses as the tuberculous animals. The results of the treatment are shown in table 1. The average loss in eosinophiles in the treated guinea pigs for the six months after treatment was 21 per cent while the average gain in the untreated for the same time was 46 per cent. The average difference was therefore 67 per cent.

We may conclude that paranitro-orthoacetoxymercuri-monomethylaniline has the effect of inhibiting the increase of eosinophiles which usually

TABLE 1

Showing the effect of treatment with No. 29 on the eosinophiles of nontuberculous guinea pigs

TREATMENT NO. 29	ANIMAL	AVERAGE NUMBER OF EOSINOPHILES		GAIN OR LOSS
		Six months before	Six months after	
		per cmm.	*per cmm.*	*per cent*
Started June 1	No. 1	235	133	44 loss
Started June 1	No. 45	465	315	35 loss
Started June 1	No. 46	510	610	16 gain
No treatment	No. 3	40	110	64 gain
No treatment	No. 20	248	345	28 gain

Average loss in treated animals in last 6 months = 21 per cent.
Average gain in untreated animals in last 6 months = 46 per cent.
Average difference = 67 per cent.

occurs in chronic tuberculosis and also decreases the high eosinophile count in animals possessing Kurloff bodies.

Four of the other drugs under consideration were mercury salts: no. 27L.C., no. 56, mercurized methylene blue, and mercuric chaulmoogric acid. In all of these cases the course of the tuberculosis was acute; in this condition we would expect the eosinophile counts to be minimal, which they were. Some nontuberculous animals with Kurloff bodies were treated with the mercuric chaulmoogric acid and they displayed the usual high eosinophile count of about 200 cells per cmm. of blood. It cannot be concluded, then, that the mercury moiety is the inhibiting agent.

2: Methylene blue: Ten tuberculous and 3 nontuberculous animals, which were being treated with methylene blue, were examined. The

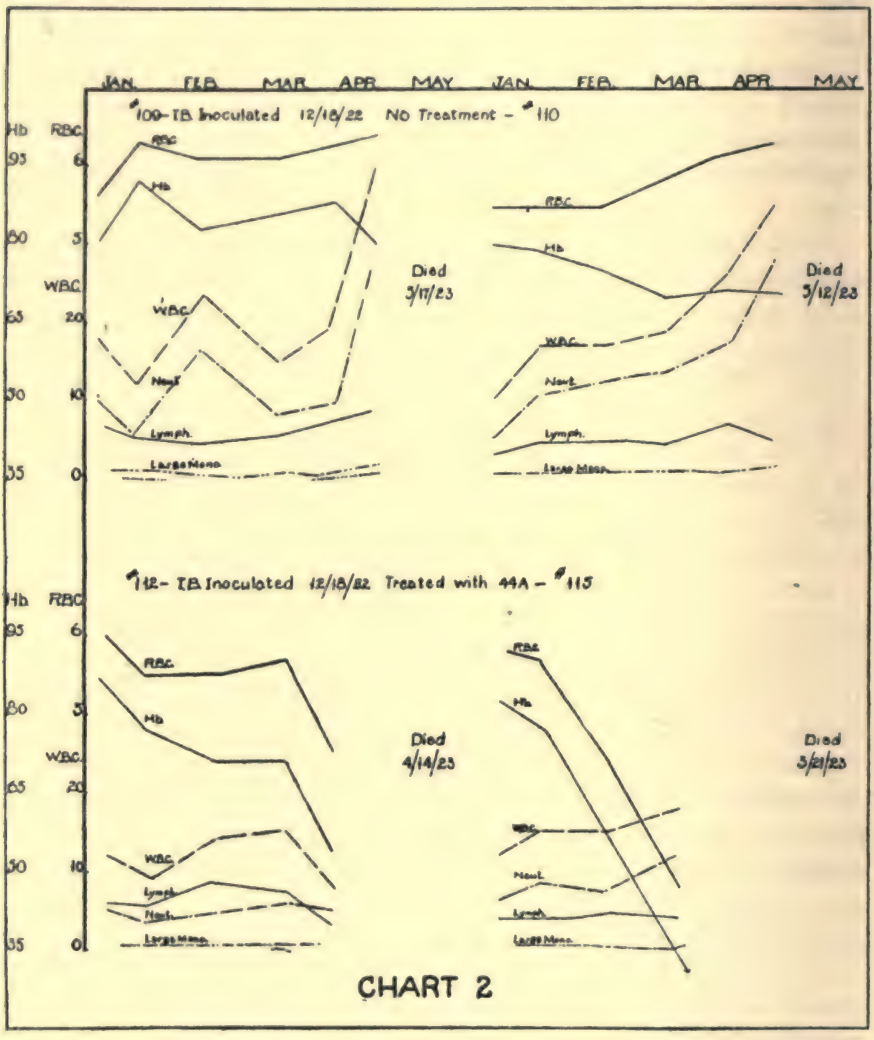
inoculated guinea pigs had acute tuberculosis, and most of them died of the disease or of acute secondary infections before many examinations could be made. Thirty-five examinations were made on the 10 animals, 9 of which were made on one animal. The leucocyte counts of these guinea pigs were high, but a leucocytosis was prevailing among all of the laboratory guinea pigs at the time, and these animals which were being treated with methylene blue in no way differed from the other acutely tuberculous guinea pigs. It appears that methylene blue had no effect on the blood picture in acute tuberculosis or in the uninoculated guinea pigs.

3: Mercuric chaulmoogric acid: Ten tuberculous and 3 nontuberculous animals were treated with this drug. The disease was of the same type as in those animals that were being treated with methylene blue just discussed. Blood pictures were similar, showing the prevailing leucocytosis. The drug appeared to have no effect on the blood picture of either the tuberculous or the nontuberculous animals.

4: No. 44A. Mercurized methylene blue: Blood examinations were made on 8 animals that were being treated with this drug. Five nontreated tuberculous animals were also examined as controls. A total of 41 examinations was made. Acute secondary infections caused the death of all but 2 guinea pigs of each set. Acute generalized tuberculosis was the cause of death in the latter. It will be seen from chart 2 and table 2 that the treated animals showed a more severe anemia than the nontreated, and that in those which survived the acute infections and died of tuberculosis the hemoglobin reached the levels of 52 and 30 per cent, the latter being the lowest hemoglobin percentage on record in our tuberculous guinea pigs. The number of erythrocytes also reached a low level, the lowest figure being 2,800,000 in the treated as compared to 5,200,000 in the untreated. The leucocyte count of the treated animals never reached the high level of the untreated animals, the maximum count being 18,000 in the treated and 40,000 in the untreated. Since the average length of life in the treated was shorter than the untreated (2.23 months as compared to 3.13 months), and autopsies showed that acute secondary infections were apparently equally severe in both cases, it would appear that the low leucocytosis was not due to less infection in the case of the treated animals but to an inhibited reaction to the infection.

Thus, mercurized methylene blue seemed to act as a poison to the blood, and especially to those elements which are formed in the bone marrow. That this action was not due to the mercury is shown by the

results of no. 29, no. 56, no. 27LC, and mercuric chaulmoogric acid, none of which had any effect on the erythrocytes or the leucocytes. Methylene blue itself did not inhibit leucocytosis or increase the anemia.



5: New methylene blue GG: Fourteen examinations were made on 5 animals which were being treated with new methylene blue GG. All of these succumbed to secondary infections. The tuberculosis, as far as it had developed was acute. There was no evidence that the treatment in any way affected the course of the blood changes in the disease.

6: Neutral red: Eight examinations were made on the 5 guinea pigs which were being treated with neutral red. These animals had acute tuberculosis but died of acute secondary infections, and the blood pictures revealed no changes due to the drug.

7: No. 56. 4-hydroxy-3-5-diacetoxymercuri-trimethylphenylammoniumacetate and *no. 27L.C. Mercuri-bis-3-3'hydroxy-5-5'trimethylphenylammoniumacetate:* Animals treated with these drugs were examined by Miss Ruth McKinney. Five animals were treated with no. 27L.C. and five with no. 56, and there were 3 treatment controls for each set. There were 4 tuberculous controls for both sets. The examinations covered a period of five months. In neither set could Miss McKinney find any difference in the blood picture due to treatment.

TABLE 2

The effect of mercurized methylene blue on the blood picture in tuberculous guinea pigs

	NUMBER OF ANIMALS	AVERAGE LENGTH OF LIFE	AVERAGE TERMINAL W.B.C.	AVERAGE TERMINAL R.B.C.	AVERAGE TERMINAL Hb.
		months			*per cent*
Treated	8	2.23	10,000	4,550,000	64
Untreated	5	3.13	25,000	5,880,000	75

CONCLUSIONS

1. No effect on the blood picture in tuberculous animals nor in nontuberculous controls could be demonstrated with treatment by methylene blue, mercuric chaulmoogric acid, new methylene blue GG, neutral red, no. 56, or no. 27L.C.

2. No. 29. Paranitro-orthoacetoxymercuri-monomethylaniline prevented the development of an eosinophilia in guinea pigs with chronic tuberculosis and decreased the eosinophile count in nontuberculous animals with Kurloff bodies.

3. No. 44A. Mercurized methylene blue seemed to act as a poison on those elements of the blood which are produced in the bone marrow, increasing the anemia and inhibiting leucocytosis in acutely tuberculous animals with secondary infections.

REFERENCES

(1) Bender, L., and DeWitt, L. M.: Amer. Rev. Tuberc., 1923, viii, 138.
(2) De Witt, L. M.: J. Infect. Dis., 1922, xxx, 365.
(3) De Witt, L. M.: Amer. Rev. Tuberc., 1923, viii, 234.

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CHEMICAL EVIDENCE ON THE PHYLOGENETIC CLASSIFICATION OF THE TUBERCLE BACILLUS[1, 2]

THE PLANT OR ANIMAL QUESTION

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Bacteria, largely on the basis of two properties, namely, spore formation and marked capability in the direction of chemosynthesis, both of them recognized characteristics of plant life, have been generally classified as members of the vegetable world. To be sure, the spores do not bear the same relation to reproduction in bacteria as in plants, but there is a superficial resemblance. Exceptions have been made with respect to certain "higher bacteria." The question of their classification has recently had some airing in *Science*. One investigator would limit plants to "those organisms possessing chlorophyl or descended from chlorophyl-possessing ancestors." Bacteria, with the exception of certain filamentous forms, are thus definitely excluded. Presumably this definition (1) does not mean that bacteria be shifted from the position they have usually held in general opinion, as forms of plant life to be classified forthwith among the animals. The division of Nature into Animal, Mineral and Vegetable Kingdoms may leave more sovereignties unspecified than

[1] From the Department of Pathology of the University of Chicago, and the Otho S. A Sprague Memorial Institute.

[2] The analysis attempted in this paper has developed out of studies on the chemistry of the tubercle bacillus, which for several years have been pursued under a grant from the National Tuberculosis Association. In 1921 the Association, on the recommendation of its Committee on Medical Research, appropriated a sum of money for the furtherance of investigations, then being conducted by the author, on the nutrition of the tubercle bacillus. In the course of these investigations it became highly desirable to obtain exact information on the nature of the nucleic acid present in the tubercle bacillus. The well known researches of Dr. Treat B. Johnson on nucleic acid chemistry led the author to ask him to study this phase of tubercle bacillus chemistry, and to devote part of the appropriation, assigned by the Association, to this work. The important researches on tuberculinic acid by Johnson and Brown, from the Sheffield Chemical Laboratory of Yale University, referred to in this paper, were made possible by this financial aid. They have been reported, in part, in the *Journal of Biological Chemistry*, December, 1922, and the AMERICAN REVIEW OF TUBERCULOSIS, June, 1923, and are being continued, and will be reported by them in detail later.

the Kingdom of Heaven, to which the puzzled child assigned the fairy tale prince. Fitting living beings into systems seldom leads to results objection-free. We have left far behind the day when one could say comfortably with Linnaeus that plants live and grow, while animals live, grow and feel.

Discovery makes for complexity more often than the reverse. Once a division of the universe into organic and inorganic matter was easy; until Friedrich Wöhler transformed ammonium isocyanate, a compound belonging to the world of nonliving things, by simple fusion and resultant intramolecular rearrangement, a procedure in which matter was neither gained nor lost, into the well-known derivative of life processes, urea. The Gordian knot tied thereby in the classification of chemistry was not solved until an official committee of chemists rudely severed it by arbitrarily ruling that all compounds containing the element carbon were *organic*, and all the rest *inorganic*. In these later days, however, when specific atoms have been divided into nonspecific electrons, and chemical elements are polymers of something common to all, a distinction based on the individuality of an "element," is in danger of not distant collapse.

Solution of the plant vs. animal controversy has been sought in some instances[3] in the establishment of a separate class, the protista, intermediate between plants and animals, or distinct from either. This may seem a logical escape from a vexed problem, and yet, in addition to its despairing aspect, serves to divert attention from interesting relationships of considerable evolutionary significance between living forms in question and one or the other of the two great groups. A very useful purpose is served by the effort to fit all living beings into one or the other class in so far as the evolutionary point of view is maintained, in that attention is kept focused on the root of all biological problems.

If Linnaeus's psychological distinction is unfit and the methods of morphological differentiation are inadequate, there remains yet another basis on which bacteria may be classified with respect to plant or animal tendencies. This is a chemical one. Wells (2), in his "Chemical Pathology," refers twice to the possibility of distinguishing bacteria as plants or animals on this basis. One means is the determination of the type of nucleic acid present, nucleic acids being divided at present into plant and animal types; the other is in the finding of such typical plant products as cellulose or hemicellulose, on the one hand, or the animal sub-

[3] Enough for the establishment of a journal, the *Archiv für Protistenkunde*.

stance *chitin*, familiar in the shells of crustaceans and wings of insects, on the other. Chitin is indeed not limited in its distribution to the animal kingdom, as many fungi of undoubted plant affiliations contain a certain amount of it (3) (4); but its content in certain animal tissues is striking. Additional chemical sources of differentiation are not wanting, even though an inquiry, comprehending all the fields within which a distinction may be made, must meet with but infrequent reward and leave many gaps, because of the sketchiness of our present knowledge of bacterial chemistry. Enough knowledge is at hand for a good start, however.

It so happens that the chemistry of the tubercle bacillus has been investigated more extensively than that of any other bacterium. If any microörganism is at present able to supply us with chemical information useful in the classification of bacteria, it is the tubercle bacillus. In the pages to follow the composition and metabolism of this bacillus will be examined with respect to this problem.

The following very abbreviated classification of the materials entering into the make-up of living things, and of the processes by which living things are made up, will serve as an outline for the consideration below of what is known concerning the tubercle bacillus which might apply in the problem under consideration.

Composition

Proteins	*Carbohydrates*	*Lipins*	*Water*
Simple	Monosaccharides	Alcohols	*Salts*
Conjugated	Polysaccharides	Fatty acids	
Nucleoprotein		Waxes	
Glycoprotein		Phosphatids	
Derivatives			
Amino acids			

Metabolism

Energy production	*Carbon metabolism*	*Nitrogen metabolism*
Oxidation	Absorption	Absorption
	Synthesis	Synthesis
	Excretion	Excretion

PROTEINS

Our discussion properly begins with the subject of proteins, because for our purpose, which is essentially a study of race, and therefore heredity, proteins are the most important. In nucleated cells it is conjugated

protein which carries the hereditary characteristics. Chromosomes consist largely of nucleoprotein. By analogy, nucleoprotein may be believed to carry the hereditary characteristics in non-nucleated forms. Proteins are highly complex and infinitely variable. Uniting them with nucleic acid to form nucleoprotein must confer further biological individuality upon them. Therefore, in an effort to separate forms of life on a chemical basis, a knowledge of their proteins and particularly nucleoproteins is of the most value.[4]

1. Simple Proteins and Amino Acids: Little systematic investigation of the proteins proper of the tubercle bacillus has been made. It is believed that at least half of the bacilliary body is in the form of protein, and it is known that some of this is in the conjugated form combined with nucleic acid. Probably some is conjugated with sugar as glycoprotein

TABLE 1

Amino acid content of tubercle bacillus protein

	TAMURA		JOHNSON AND BROWN	
	per cent		*per cent*	
Total nitrogen	100		100	
Basic nitrogen	11.85		37.63	
In arginine		7.64		10.63
histidine		1.53		11.48
lysine		1.49		3.69
ammonia		1.15		11.83 (amide)
Monoamino acid nitrogen	66.59		47.39	
Humin nitrogen	15.79		4.11	

also, but this has not been well worked out. The nature of the protein present is also obscure. Some work indicates that albumen and globulin may be present, and Ruppel claimed that he found a protamine, but the results lack confirmation—as well as refutation.

More attention has been paid to the cleavage products of the proteins. Tamura (5) in 1913 and Johnson and Brown (6) recently, have published analyses of the tubercle bacillus for amino acids. These differ considerably, however, as shown by table 1. High percentages of the monoamino acids, phenyl alanine and valine, were conspicuous findings of Tamura, while the histidine figure of Johnson and Brown is noteworthy.

As a matter of fact, there is nothing highly characteristic of the amino acid content of plant proteins or animal proteins. The proteins of sperm

[4] For a very interesting article on the evolution of the proteins the reader is referred to Kennaway, *Chemical News*, 1920, cxx, 13.

heads, which are unusually rich in the diamino acids, particularly arginine, constitute an exception to this statement. It is commonly found, also, that vegetable proteins have a high content of glutaminic acid. Furthermore, occasionally they lack one or more of the common amino acids entirely, but this is true of some animal proteins also. The glutaminic acid content of the tubercle bacillus has not been determined. The amino acids which are occasionally lacking in proteins, as lysine and tryptophan in zein, and tyrosine in gelatin, are all present in the tubercle bacillus. Only one common protein is known to have as high a histidine content as that reported by Johnson and Brown for the tubercle bacillus. This is the animal protein globin (from hemoglobin) (7). The similarity here may be coincidental, but calls for more work on this important point. In general, it may be said that there is nothing known in the character of tuberculoprotein which would relate it particularly to one type or the other.

2. *Conjugated Protein and Nucleic Acid:* On the other hand, the best evidence we have at present for classifying the tubercle bacillus in either group comes from the analyses of tuberculinic acid, the nucleic acid of the tubercle bacillus. The knowledge here referred to we owe to the recent researches of Johnson and Brown (8). For proper understanding of the significance of their results a few words are necessary concerning the chemistry of nucleic acid. A full treatment will be found in the monograph on this subject by Walter Jones (9). A half century of research, following the pioneer investigations of Miescher on nuclein, has established the fact that α-nucleic acids are well defined compounds of characteristic composition, into which the following substances regularly enter: phosphoric acid, a carbohydrate and two purine bases and two pyrimidine bases. Furthermore, the tendency of modern research is to recognize but two α-nucleic acids, in view of the fact that the best analyses of nucleic acids reported seem to identify all of them with one or the other of two types, of which yeast-nucleic acid and thymus gland-nucleic acid, respectively, are representative members. The following schematic representation of the composition of these two acids, taken from Levene and Jacobs (10) (11), shows the differences in their make-up:

Yeast nucleic acid	*Thymus nucleic acid*
Phosphoric acid-pentose-guanine	Phosphoric acid-hexose-guanine
Phosphoric acid-pentose-adenine	Phosphoric abid-hexose-thymine
Phosphoric acid-pentose-uracil	Phosphoric acid-hexose-cytosine
Phosphoric acid-pentose-cytosine	Phosphoric acid-hexose-adenine

It is seen that the two acids are similar in structure, but display two important differences: (1) the carbohydrate linkage, between the phosphoric acid on the one hand and the characteristic bases on the other, is in the case of the yeast acid a *pentose*, and in the thymus acid a *hexose;* and (2) the pyrimidine base *uracil* appears in the yeast nucleic acid and not in the thymus form, while the pyrimidine base *thymine* appears in the thymus but not in the yeast acid.

"These two nucleic acids," says Jones,

> are the best known members of two sharply defined groups into which all substances of this class may be divided, and indeed there is a well formed and constantly growing opinion among physiological chemists that all nucleic acids are identical with one or the other of these two compounds. . . . . If such literature with its unavoidable errors be reviewed in the light of our most recent knowledge, all confusion disappears, and we come to understand that there are but two nucleic acids in nature, one obtainable from the nuclei of animal cells, and the other from the nuclei of plant cells. In examining the evidence one naturally takes into consideration that experimental exactness in such a field is entirely out of the question and that concurrent tendencies are all that can be reasonably demanded.

The chief objection to these conclusions is that the number of nucleic acids so far examined is small. In the group designated as *plant nucleic acids* we have but two well investigated members, which indeed seem identical; these are *yeast nucleic acid* and the nucleic acid of the wheat embryo, *tritico nucleic acid*. In the animal group a larger number have been extensively investigated, including thymus, pancreas, spleen and various kinds of sperm acids. Until we have a larger representation of well analyzed members of the plant group, agreeing in their make-up with the analyses of the present well investigated forms, the significance of bacterial analyses with respect to plant and animal classification will naturally be subject to some doubt.

However, on the basis of present existing knowledge and belief, we have definite evidence permitting us to classify tuberculinic acid. Johnson and Brown, working with 803 gm. of dry tubercle bacilli in two lots,[5] one of which consisted of human tubercle bacilli and the other of bovine and human types mixed, *isolated 7.7 gm. of pure nucleic acid, which on acid hydrolysis yielded cytosine and thymine but no uracil*. Since the

[5] Supplied by Parke, Davis & Co. and the H. K. Mulford Company, as byproducts of tuberculin manufacture.

publication of their article, Brown, who is continuing the study, has repeated the investigation on 810 gm. of dry bacilli with identical results.

Tests to ascertain the nature of the sugar present indicate that this answers every qualitative test for a hexose and not a pentose. It is to be mentioned that Bendix (12) obtained, on the hydrolsis of tubercle bacilli and also on hydrolysis of the nucleoprotein obtained from them, a reducing substance which yielded an *osazone* and an *orcin* addition product which were apparently of the pentose type. Brown's product, however, that is to say, *nucleic acid as pure as modern methods will make it*, freed from all protein and extraneous matter, yields formic and levulinic acid and only a small amount of furfuraldehyde on acid hydrolysis. This result speaks for the presence of a hexose, not a pentose.

The other constituents of the nucleic acid of the tubercle bacillus have been isolated and identified. The phosphorus constitutes 8.2 per cent of the acid, according to a figure obtained by Brown on a part of his material. This is not far from the figure of 8.9 per cent demanded by the Steudel and Levene and Jacobs formulas. Long (13) isolated both *adenine* and *guanine* in almost equal quantities and could not find any other purines present.

Altogether, while no attempt to determine the configuration of the several substances isolated from tuberculinic acid has been made, enough work has been done to indicate that the usual constituents of a nucleic acid are all present, and that these substances are such that tubercle bacillus-nucleic acid corresponds with the animal type and not the plant. However vague and confusing other data with regard to the phylogenetic classification of the bacillus may be, this one fact stands out with exceptional clearness. It should be added that no other bacterial nucleic acid has been so well investigated.

CARBOHYDRATES

Unfortunately, no systematic study of tubercle bacillus carbohydrates comparable to the investigation of the nucleic acid has been made. As mentioned above, monosaccharides are constituents of nucleic acid itself, and a discussion, concerning that part of the carbohydrate of the tubercle bacillus entering into the composition of nucleic acid, is given above. Besides this moiety, sugar-like substances are to be found in several other fractions of the tubercle bacillus. Carbohydrate is probably present as part of protein molecules, as part of lipin molecules, and also in un-

combined form. Agulhon and Frouin (14) obtained from tubercle bacilli a phosphatid which they believed to belong to the class of jecorins, as it liberated on hydrolysis, besides fatty acid, glycerol and a base analogous to *choline*, a gummy polysaccharide from which glucose split off on further hydrolysis. This finding has not yet been confirmed by repetition. This compound will be referred to again in the discussion on phosphatids below.

One of the best means for differentiating plant and animal forms should be found in the field of the polysaccharides. As a matter of fact we are almost uninformed as to the type of compound of this class present. To be sure there are reports of substances of the soluble glycogen-inulin type, and again there are claims for the presence of such insoluble polysaccharides as cellulose and the nitrogenous "carbohydrate" chitin, a polymer of glucosamine. Of the substances mentioned, a content of glycogen or of chitin would speak for an animal form, while the presence of inulin or cellulose would relate the microögranism quite definitely to the plants.

There is an old report of a compound like glycogen in the body of the bacillus. Levene (15) extracted a substance which remained in solution after removal of nucleic acid and protein. It gave an opalescent solution in water, and could be precipitated with alcohol and basic lead acetate. With iodine it gave a color test similar to that obtained with glycogen. It did not reduce Fehling's solution directly but did so after acid hydrolysis.

Panzer (16) has also reported the presence of a water-soluble polysaccharide. He extracted a substance free from phosphorus, nitrogen and sulphur, which gelatinized on cooling. It was precipitated by alcohol and lead acetate and reduced Fehling's solution after hydrolysis. The substance failed to liberate mucic acid on hydrolysis (test for galactose) and was therefore not a galactan. Physically, it resembled gum arabic, which is a pentosan, and, it should be added, a peculiarly plant substance. The hydrolysate gave the Molisch α-naphthol test, but this is of no significance in distinguishing pentoses from hexoses. Panzer's material was far from sufficient to enable him to tell whether the substance was really made up of pentoses or not, and apparently no attempt to determine this was made.

More ambitious was the study of Tamura (17), who also, and with much more evidence, concluded that pentosans were present. His experiments were made with the tubercle bacillus and also with an acid-

fast saprophyte, *Mycobacterium lacticola*. After separation of fatty substances, and as complete removal as possible of the proteins, he had left a substance which was insoluble in alkali, insoluble in ammoniacal copper solution (Schweitzer's cellulose reagent), failed to give the iodine reaction for chitin, and was easily hydrolyzed by 3 per cent sulphuric acid, the hydrolysate reducing Fehling's solution readily and giving the orcin hydrochloride test for pentoses. An osazone was obtained from the *Mycobacterium lacticola* material, which on analysis gave figures corresponding to the pentose arabinose. The tubercle bacillus product, which resembled the mycobacterium product in all other respects, was insufficient for this test. From these results Tamura concluded that chitin and cellulose were absent, and that he was dealing with a hemicellulose or pentosan.

These results furnish a strong argument for the vegetable nature of the bacillus, as pentosans are typically plant products. However, several objections must be made to Tamura's conclusions, with due recognition of the excellence of the work done. In the first place, aside from the fact that his best figures come, not from the tubercle bacillus, but from the *Mycobacterium lacticola* material, his yield of carbohydrate was very low. His biggest yield was from mycobacteria, defatted but not treated for removal of proteins. From 50 gm. of dry bacterial body he obtained 0.1 gm. of what appeared to be *arabinose hydrazone*. Granting that his product was truly a pentose, it must still be admitted that this quantity could be locked up in some other combination than pentosan. So, too, the defatted, alkali-treated material, referred to in the preceding paragraph, was not necessarily pentosan merely because it contained pentose. Tamura himself states that by alkali treatment it was impossible to free it completely from protein. Pentoses, which were first found in the animal kingdom, are present in β-nucleic acids, such relatively simple compounds as the guanylic acid of the pancreas, and perhaps elsewhere. Clinicians will be reminded of this fact in the Cammidge test for diseases of the pancreas, and the occasional occurrence of persistent pentosuria in man. The significance of Tamura's findings is thus not sharp, but it must be admitted that they are important and that they constitute the best evidence available to-day for classifying the tubercle bacillus with the plants on the basis of chemical composition.[6]

[6] Baudran (18) claimed that he found true cellulose in the tubercle bacillus, but his contention is based largely on a color reaction and is not convincing.

The search for carbohydrates of characteristically animal nature has led to practically negative results. To be sure Helbing (19) compared the acid-fastness of the tubercle bacillus to that of the shell of certain taenia eggs known to contain chitin, and suggested that the acid-fastness of the tubercle bacillus might be due to chitin. No experimental proof accompanied the suggestion, however. On the other hand, definite attempts to isolate *glucosamine*, the characteristic hydrolytic product of chitin, have failed (20).

Thus, summing up our knowledge on the carbohydrates of the tubercle bacillus, we find the best evidence to be in favor of a classification in the plant world. The evidence is meagre enough, however, and objection can be raised to all of it. The situation distinctly calls for more work, and until that is forthcoming, here, in this unsettled way, the case must rest.

LIPINS

1. Wax and Alcohol: The lipin content of the tubercle bacillus has undoubtedly been more extensively investigated than all the other fractions together. The strikingly high lipin content was impressive to the earliest observers. A critical examination of the known facts might throw light on the question here under consideration. The 30 to 40 per cent of the bacillary substance which is soluble in such reagents as ether, chloroform, ligroin and toluene, includes some free fatty acids, some fats, some phosphatids, but chiefly (60 to 70 per cent, Long and Campbell (21)) a waxy substance which is an ester of fatty acids with an alcohol of high molecular weight. It is upon this wax that interest has chiefly centered, and its nature is by no means yet revealed. The tendency is to accept Tamura's analysis (5) as probably the closest approximation on record. This observer believed it to be the lauric acid ester of an alcohol of the composition $C_{29}H_{55}OH$. There are indications that other acids than lauric are present, however.

What light does the presence of this compound shed on the problem which we are discussing? Bürger (22) believes that the lipin resembles plant waxes, and points out that such cerolipoids occur commonly in the tissues of xerophytic plants and are protective in nature. However, he adduces no evidence to show that the characteristic part of tubercle bacillus wax, that is, the alcohol or sterol (solid alcohol) part, resembles phytosterols more than zoösterols, if we may introduce the latter term. It appears to be indeed true that the commonest animal sterol, choles-

terol, is absent from the tubercle bacillus, but the physical similarity of mycol (tubercle bacillus—and probably other bacterial—sterol) to cholesterol is close enough to have kept the search for the latter in the bacillary substance active for some time. Certainly, substances similar to mycol are widely distributed through the animal kingdom. Czapek (3), in his comparison of plant and animal sterols, is able to present no chemical means of classifying them in two groups. He describes differences between individuals of the two series, such as in melting point and crystalline form, but there appears to be nothing absent from one group and holding good throughout the other, which would serve for differentiation of unknowns. Altogether, important as it is in the make-up of the tubercle bacillus, mycol furnishes us with no basis whatsoever for plant or animal distinction.

2. *Phospholipins:* Phospholipins are widely distributed throughout nature and are not absent from the tubercle bacillus. As is well known, phospholipin problems are among the most difficult in all physiological chemistry. It is only in the last few years, thanks largely to the brilliant work of MacLean and Levene and their associates, that order is at last coming out of chaotic confusion. Even yet, perfect agreement as to the chemical composition of the best known and best studied members of the group is by no means reached. The battle still rages over the actual existence of other substances. The reason for this lies in the extreme difficulty in purifying bodies of this class and in their instability.

Claims in the field of phospholipins, to be convincing, must be based on repeated observation, rigid purification of material and a wealth of chemical experience, in addition to the obvious requirement of original capacity on the part of the investigator. This means years of work and an abundance of material. It is small wonder then, that much significance cannot be attached to the few isolated observations concerning the slow-growing tubercle bacillus in this difficult domain. There are several observations on record, however, and these might be of much significance as to the problem in hand, could we take them at their face value.

Before considering these claims, a few words should be said concerning the distribution of phospholipins in nature. First isolated from animal tissues they were generally thought of as animal products until, after a time, reports of "plant lecithins" began to be made. Since then it has come to be believed likely that substances of this general class are pres-

ent in all cells, in which they play important rôles in oxidation and also in cell permeability. However, there is still a more or less widely spread impression that although the number of main groups of phospholipins is not large, small differences within the molecule may go far toward conferring biological individuality.

Widely distributed as plant phospholipins may be, and complex and variable as subsequent research may prove them, it is unquestionable that they reach their greatest development and are present in greatest proportion in the most animal of all animal tissues, the central nervous system. It is here that they are most easily obtained in quantity and have been most extensively and most creditably investigated. The finding in the tubercle bacillus of lipoids like the brain lipoids can, at least, never support the hypothesis of plant affiliation.[7] Whether or not it supports the animal hypothesis depends upon two things: first, upon the certainty of the correctness of the finding and, second, upon what future research tells us concerning the existence of the same compounds in the plant world.

The most recent reported investigation of the lipoids of the tubercle bacillus, which should have had the advantages of modern knowledge, is that of Koganei (23) of Tokyo. Unfortunately, in this report the chain of reasoning is most difficult to follow. A few short analyses lead to the abrupt, unexplained conclusion that *phrenosin*, *kerasin*, *sphingomyelin* and *kephalin* are present. A fair sample of the character of the work is seen in the claim for the presence of sphingomyelin on the basis of a 1:1 nitrogen-phosphorus ratio, and the fact that on baryta hydrolysis only choline and phosphoric acid went into solution. The identification of the base is most gravely open to question. The addition of platinum chloride to the hydrolysate caused a precipitate "which showed definitely the presence of choline." Without going deeply into the chemistry involved, it suffices to say that many things besides choline are precipitated by platinum chloride, that choline and phosphoric acid could just as well be split off from egg lecithin by his method, and finally that the N:P ratio in sphingomyelin is not 1:1 but 2:1.

The identification of kephalin is equally lucid and convincing. A substance admitted by the author to be four-fifths impurity gives nitro-

[7] An able plant physiologist, who has had the kindness to look this paper over and delete its most glaring mistakes, makes the following comment on this sentence, "The botanist would insist that the discovery of such compounds in fungi (including bacteria) would establish the presence of compounds of this class in plants."

gen and phosphorus figures which signify that "there is no doubt about the presence of kephalin." The acid-fastness of the tubercle bacillus is rashly but definitely attributed to kephalin, and the author concludes triumphantly with the statement that the four substances phrenosin, kerasin, sphingomyelin and kephalin have never before been recorded as constituents of tubercle bacilli.

It is unfortunate that effort has not been more wisely expended in this field. The importance of the phospholipins warrants the most intelligent consideration. It is perhaps not exceeded by that of the well known wax.

A finding which may have some slight bearing on the problem is that of Agulhon and Frouin (14), who isolated a compound hydrolyzing into glycerol, fatty acids, a base analogous to choline, and a gum which on further hydrolysis yielded glucose. The substance was classified as a *jecorin*, from its similarity to the jecorins so widely distributed in animal tissues. Jecorins probably have no status as chemical units. They are now believed to be simply mixtures. The difficulty of removing sugar and other impurities from the phosphatids, especially from the kephalin of the brain, is well known. It should be mentioned that phosphatids from which sugar can be separated only with difficulty have been isolated and studied in plants by Winterstein and Hiestand (24).

Altogether, in going over the literature of tubercle bacillus chemistry, I find only one article on phospholipins in the bacillus to which I am willing to attach significance. Tamura (5), whose other contributions have several times been referred to above, in the course of his investigation of the tubercle bacillus in Kossel's laboratory, precipitated with acetone and carried through some stages of purification a substance which gave, with more than fair accuracy on two trials, a nitrogen-phosphorus ratio of 2:1. There is no doubt that modern technique would demand a more rigid purification than Tamura carried out and not two analyses but many. However, incomplete as it is, it represents the best analysis of a tubercle bacillus phosphatid that we have. It suggests the presence of sphingomyelin or a related compound. Sphingomyelin appears to have a wide animal distribution. Levene has obtained closely agreeing figures with products from brain, kidney, liver and egg yolk. Whether or not it has plant distribution remains for future research to show.

3. Fatty Acids: We do not need to devote much space to these compounds. What knowledge we have is fairly definite. *Palmitic acid* was found in the bacillus by the early investigators. This acid has a wide distribution in both kingdoms. *Oleic acid*, also found, likewise characterizes neither. *Lauric acid*, on the other hand, which Goris (25) and others have found in tubercle bacilli, appears to have a wider distribution in plants than in animals, deriving its name from its abundance in the laurel berry. Beilstein's *Handbook* may be consulted for a summary of the vegetable substances in which it is found. It occurs also in many animal products, although apparently in most instances simply as a result of its presence in the animal's diet, with absorption and storage in unchanged form. In such fashion, also, we may readily explain its presence in milk. For our present purposes the type of fat in a given substance is significant only if the fatty acids were synthesized there. In tubercle bacilli grown on glycerol-broth the lauric acid, which the researches of Goris (25) and others have found in considerable quantity, must have been synthesized by the bacilli. The same may be said of the plant fatty acid, *crotonic acid*, which Goris claims exists in a glassy product from the bacillus, which he designates *hyalinol*.

Lauric acid occurs in some animal products, in which its presence is not so readily explained as a dietary sequel; for instance, in spermaceti, the wax obtained from the skull cavity of the sperm whale. It is interesting that in this substance lauric acid exists in a combination similar to that found in tubercle bacilli, namely, as the wax-like ester of an alcohol of high molecular weight. Such facts are sufficient to withhold us from including the presence of lauric acid among the definite plant characteristics.

METABOLISM

It is partly on the basis of metabolism that the tubercle bacillus and other bacteria have been generally classified among the plants. An ability to exist on simple compounds, and transform these readily into complex protoplasm, is supposed to be one of the distinguishing attributes of vegetable life. If the tubercle bacillus were not heterotrophic, and were able to withdraw carbon dioxide from the air and grow with this as its sole source of carbon, there would be little or no opposition to placing it forthwith among the plants. An examination of the known facts of its metabolism will show that the situation is not so simple.

Several distinctive features of the metabolism are known and fairly well understood. Some of these have been discussed in these pages before.

1. Nitrogen Metabolism: The tubercle bacillus, like the majority of bacteria, will thrive when nitrogen is furnished in very simple form. So far from requiring ready-built proteins, proteoses or peptones, it can satisfy its nitrogenous needs with amino acids, or a single amino acid if necessary, or, finally, even with ammonia alone. It synthesizes purine bases and the cyclic amino acids when the only nitrogen supplied is ammonia (13). It is helpless only when its nitrogen supply is limited to nitrates, nitrites or elemental nitrogen. This capacity has been considered peculiarly plant-like, and it must be admitted that the synthesis of proteins from ammonia and organic matter is one of the great functions of plant life. This endowment, with its sister one of reducing carbon dioxide, saves the living world from inevitable decay. But that it is peculiar to plant life is another matter. It is unquestionable indeed that the higher forms of animal life take their nitrogen in a more complex state than ammonia: amino acids and a considerable variety of them, are usually required. Conversely, they could not live, were they rationed with ammonia as their sole source of nitrogen. However, it is not so certain that they are unable to synthesize amino acids or use ammonia at all. Certainly, one amino acid can be liberated in the body of herbivora in huge quantity, probably in excess of any possible stored amount and must therefore be synthesizable. I refer to the liberation of *glycine* under the stress of detoxicating large amounts of benzoic acid. In addition, I think it has been satisfactorily demonstrated that the reaction by which the amino acid *alanine* is broken down into pyruvic or lactic acid and ammonia is a reversible one, namely, that the amino acid can be synthesized from these substances by the tissues of even the fastidious mammalian body. Embden and Schmitz (26) were able to recover alanine (in excess of that formed by autolysis) after perfusion of the isolated liver with ammonium pyruvate, and demonstrated a similar synthesis with ammonia and several oxyacids. This evidence is cited, not with the intention of suggesting that such a process is a common type of metabolism, but simply to prove that, far removed as they are from plant forms, some, at least, of the mammals have retained from some distant ancestor's possessions what must have been a fundamental property, that of synthesizing very simple nitrogenous

substances into complex ones. On the whole, the extensive capacity manifested by the tubercle bacillus in the utilization of nitrogen is unlike anything commonly seen in the order of higher animals and quite like that seen in both the lowest and highest plants.

Ammonia may also be looked upon as an excretory product of the tubercle bacillus. Recognized plants similarly break down their own proteins and liberate some ammonia. But this is no distinctive property. Animals, including man, possess it. Prone as they are to excrete nitrogen in some more complicated form, as urea or uric acid, under certain conditions, particularly the necessity of neutralizing acid, higher animal organisms, as well as lower, are capable of liberating considerable amounts of ammonia from their tissues.

2. Carbon Metabolism: I have said that the capacity for synthesizing complex compounds from very simple substances is characteristically a plant one. The green plants need only nitrates for nitrogen, carbon dioxide for carbon, and water and certain salts, to carry on existence. The higher animals practically require, in addition to water and the same salts, amino acids for their nitrogen, and make extensive use of sugar and fats. As far as carbon utilization is concerned, the tubercle bacillus is unique in one way. Of course, devoid, as it is, of chlorophyl, it makes no use of carbon dioxide. But it is particular in its requirements beyond this. As is well known, most sources of carbon do not satisfy the bacillus. It will utilize certain sugars slowly, and I have seen it propagate feebly and for a limited period on certain acid amides; but for steady and luxuriant growth, what we may call normal growth, glycerol is essential. It seems to function partially in forming the wax in which the bacillus is so rich. It seems to me that this inflexibility, this failure to be satisfied with the simple acids, such as acetic, propionic, glyoxylic, lactic or pyruvic acid, or a simple alcohol, like ethyl alcohol, which, by the way, do support many bacteria, is very interesting, but it offers no means for distinction from the heterotrophic plants.

Little needs to be said about the energy production of the tubercle bacillus; it resembles that of both plant and animal cells in that carbonaceous compounds are oxidized. Its carbon excretion, like that of all cells, is partly in the form of carbon dioxide. In addition it excretes organic acids, or perhaps forms them from the constituents of the medium, which in the "take and leave" mode of existence of unicellular forms is

analogous to the excretion of higher forms. Both plant and animal forms do this also abundantly.

A discussion of metabolism could not be closed without some reference to the vitamins, the growth accessory substances. It has been sometimes said that for plants vitamins are unnecessary and that for animals they are required. At present we are by no means sure that plants do not need them too, for continued growth. The tubercle bacillus requires more study before we can definitely state the situation with it. I have tried with commercial vitamin preparations to increase its rate of growth, and have not succeeded. On the other hand, I have repeatedly grown the bacillus in simple media containing no trace of vitamin. But it must be remembered that inoculation of media with the tubercle bacillus must always be generous. Furthermore, it is a fact that, so far from becoming adapted to growth on simple media, cultures tend to run out and ultimately stop growing if repeatedly transferred on simple, so called synthetic media. May this not be the result of an ultimate failure of vitamin? Certainly, it is doubtful if, in its vitamin relation, the tubercle bacillus displays definite plant leanings.

SUMMARY AND CONCLUSIONS

From the arguments advanced above it is at once apparent that, seen from the chemical point of view, the tubercle bacillus exhibits recognized plant tendencies and recognized animal ones. Summarized they are about as follows:

Fairly definite

For the animal:

It contains a typical nucleic acid of the animal type, probably identical with that of thymus, pancreas, sperm and spleen. It does not contain nucleic acid of the so called plant type found in yeast and wheat. The basis for these statements lies in the established presence of the pyrimidines, thymine and cytosine, in tubercle bacillus nucleic acid, and the presence of a hexose, and the established absence of the pyrimidine uracil.

For the plant:

It possesses marked chemosynthetic power in nitrogen metabolism. synthesizing such complex substances as amino acids and purine bases with no other source of nitrogen than ammonia.

Less definite

For the animal:

It is said to possess lipins of the diamino-monophosphatid type, which so far have been described convincingly only in animals.

For the plant:

It is said to contain hemicellulose or pentosans, made up of pentose units, which are typical plant substances. An appreciable content of the fatty acid, lauric acid, which has a wide plant distribution, has also been recorded.

The question may well be raised of whether it is futile to attempt to decide the question on a basis so full of conflict. The answer is that out of such an exposition of known facts, as has been attempted above, some distinctions might be found presenting possibilities for arbitrary classification. The situation in organic chemistry is recalled and the purely arbitrary but highly useful classification into great divisions on the basis of the presence or absence of carbon. I have no intention of settling the matter in this paper. For me the question remains open, until such time as future research furnishes a basis on which a distinction, purely arbitrary perhaps, may be made. I confess to the hope that in the nucleic acids such a distinction may be ultimately found. It is Johnson and Brown's finding in the tubercle bacillus of what present day research definitely characterizes as an animal nucleic acid that prompts this survey of the whole field. But I realize that the present distinction of nucleic acids as of plant and animal types rests on a slender foundation. The finding of a nucleic acid identical with tubercle bacillus nucleic acid in a pumpkin seed, for instance, would wreck my hope. On the other hand, should chemical research continue to find "plant nucleic acids" only in unquestioned plants and "animal nucleic acids" only in what are by common consent animals, I would regard the tubercle bacillus finding as one of great evolutionary significance.

I am frankly doubtful, however, whether the situation will ever prove so simple. Biological variation of protoplasm means differences, not only in constituents, but also in their relations. As Levene (27) puts it, "Specificity, individuality or character may be brought about not by single substances, but rather by definite and constant equilibria of substances." E. B. Wilson (28) writes, "When we speak of protoplasm as the physical basis of life we mean simply the sum total of all the sub-

stances that play any active part in cell life." We shall know where to put the tubercle bacillus in the kingdoms of nature when we have learned not only its composition but the interrelations of its elements. We have much to learn; but in the meantime it is to the credit of those who have added to our knowledge of the chemistry of the tubercle bacillus that they have put on record for this bacillus far more than has been done for any other bacterium.

REFERENCES

(1) MARTIN: Science, 1923, lvii, 175.
(2) WELLS: Chemical pathology, Saunders & Co., Philadelphia, 1st Ed. 1907, 4th Ed. 1920, Chap. IV.
(3) CZAPEK: Biochemie der Pflanzen, Jena, 1913.
(4) TUNMAN: Pflanzenmicrochemie, Berlin, 1913, p. 606–610.
(5) TAMURA: Zeit. physiol. Chem., 1913, lxxxvii, 85.
(6) JOHNSON AND BROWN: Jour. Biol. Chem., 1922, liv, 721.
(7) ABDERHALDEN: Zeit. physiol. Chem., 1903, xxxvii, 484, and 1907, li, 397.
(8) JOHNSON AND BROWN: Jour. Biol. Chem., 1922, liv, 731.
(9) JONES: Nucleic acids, Longmans, Green & Co., 2nd Ed., 1920, London.
(10) LEVENE AND JACOBS: Ber. Deutsch. chem. Gesellsch., 1910, xliii, 3151, and 1911, xliv, 1027.
(11) LEVENE AND JACOBS: Jour. Biol. Chem., 1912, xii, 411.
(12) BENDIX: Deutsch. med. Wchnschr., 1901, xxvii, 180.
(13) LONG: Amer. Rev. Tuberc., 1921, iv, 842.
(14) AGULHON AND FROUIN: Bull. Soc. Chim. Biol., 1919, i, 176.
(15) LEVENE: Jour. Med. Res., 1901, vi, 135.
(16) PANZER: Zeit. physiol. Chemie., 1912, lxxviii, 414.
(17) TAMURA: Ibid., 1914, lxxxix, 304.
(18) BAUDRAN: Compt. Rend. Acad. Sci., 1906, cxlii, 657.
(19) HELBING: Deutsch. med. Wchnschr., 1900, xxvi, 1335.
(20) KOZNIEWSKI: Bull. Internat. Acad. Sci. Cracovie., Series A, 1912, 942.
(21) LONG AND CAMPBELL: Amer. Rev. Tuberc., 1922, vi, 636.
(22) BÜRGER: Biochem. Zeit., 1916, lxxviii, 155.
(23) KOGANEI: J. Biochem., 1922, i, 353.
(24) WINTERSTEIN AND HIESTAND: Zeit. physiol. Chem., 1906, xlvii, 496.
(25) GORIS: Ann. d. l'Inst. Pasteur, 1920, xxxiv, 497.
(26) EMBDEN AND SCHMITZ: Biochem. Zeit., 1910, xxix, 423.
(27) LEVENE: Physiol. Rev., 1921, i, 327.
(28) WILSON: Science, 1923, lvii, 277.

Relation of Clinical to Necropsy Diagnosis in Cancer and Value of Existing Cancer Statistics

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RELATION OF CLINICAL TO NECROPSY DIAGNOSIS IN CANCER AND VALUE OF EXISTING CANCER STATISTICS

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Among the factors often mentioned as responsible for the undeniable fact that more deaths from cancer have been recorded in recent years than formerly is the increment of cases of cancer recognized at necropsy that were unrecognized during life. These added cases help to swell the cancer death rate; and hence, the more advanced the medical profession of a given community, the more necropsies, and the smaller the number of cancers that escape inclusion in the vital statistics of the community. The experience of necropsy disclosure of a clinically unrecognized malignant growth is so common that this factor is always brought forward when the alleged increase in the frequency of cancer is under discussion; and it is universally admitted. Despite the recognition and acceptance of this factor, however, we have very little in the way of actual evidence as to its real importance.

The most quoted paper is that of Reichelmann.[1] He examined the records of 7,790 necropsies at the Hospital Freidrichshain zu Berlin, from April 1, 1895, to June 24, 1901, among which there were 711 carcinoma cases, or about 9 per cent. He does not include sarcomas or forms of malignancy other than carcinoma. Among these 711 carcinoma cases were 156, or 22 per cent., that were not recognized during life as malignant disease, irrespective of whether the site or the character of the growth was correctly diagnosed. That is to say, if necropsies had not been performed in these cases, the vital statistics would have contained 555 instead of 711 cases of cancer from this group of deaths; i. e., necropsies added about 28 per cent. On the other hand, fifty-eight cases were diagnosed cancer when this condition was not present, so actually the

1. Reichelmann: Berl. klin. Wchnschr. **39**: 728 and 758, 1902.

figures are as follows: In 769 cases, cancer was either diagnosed correctly (62.5 per cent.,), missed when it was present (20 per cent.) or diagnosed as present when there was no cancer (7.5 per cent.). Subtracting the cases added by erroneous diagnosis of cancer when it was not present (fifty-eight) from the number added by missing the cancer (156), we find that the performance of necropsies actually added ninety-eight cases to the vital statistics, or about 15 per cent. Carcinoma was diagnosed correctly 555 times, and incorrectly fifty-eight times, and was missed 151 times when present, so that there were 214 errors to 555 correct diagnoses, a diagnostic error of 27.5 per cent. in the 769 cases. Reichelmann cites also a paper by Hofmann [2] based on hospital statistics in Kiel, not available to me, which showed an addition of 19.6 per cent. to the cancer figure through necropsy.

A statistical study of the cancer cases in a large number of London hospitals has been reported by Bashford,[3] who discusses the influence exerted by microscopic examination in cancer statistics; but in these figures no exact distinction is made between cases examined at necropsy and tumors removed at operation and then examined microscopically. In all such cases, of course, the cancer had been viewed and a gross anatomic diagnosis had been made. This includes most of the tumors classed as accessible in Table 1.

Even in this group of cases, microscopic study, either with or without necropsy, added 121 cases to the 683 recognized without the aid of the microscope, or nearly 18 per cent. Presumably, the inaccessible cases alone were recognized first by necropsy, and here we find 106 added to 284, or 37.3 per cent.

In view of the fact that the foregoing statistics were compiled in other countries, and at a period when the roentgen-ray examination was not available with its important diagnostic aid, it seemed worth the labor to ascertain how things stand in this respect in a material under my own observation. I have, therefore, gone over the available necropsy records of the Cook County Hospital for the years 1917-1922, inclusive, comprising about 2,712 necropsies, and also 1,000 other records of my own necropsies not included in these statistics,

2. Hofmann: Statistik der nicht diagnostizierten Krebse, Inaug. Diss., Kiel.
3. Bashford: Scientific Reports of the Imperial Cancer Research Fund. London, 1905, No. 2, Part 1.

although many of them were performed in the Cook County Hospital at an earlier date than 1917. In these 3,712 necropsies were disclosed 545 cases of malignant disease, or 14.7 per cent. Our figures cover all forms of malignant tumor, including cerebral gliomas and endotheliomas, that were met with on the necropsy table.

The summary of these records in respect to the chief question under consideration, i. e., how many cancers are recorded in the vital statistics that would have been omitted but for necropsy, is given in Table 2.

To offset this large error of omission, we find errors of commission, i. e., cancer diagnosed as present when no malignancy was found at necropsy, there being twenty-three such in the Cook County Hospital figures and ten in the University of Chicago figures, making a total of thirty-three to be subtracted from the 178 cases erroneously added. Therefore, our necropsies added to the 400 cases diagnosed cancer during life (367 correctly and thirty-three incorrectly) 145 cases, or 36 per cent. In 578 cases (367 correct, 178 omissions and thirty-three erroneously called cancer), there were 211 incorrect diagnoses, or a diagnostic error of 36.5 per cent.

When we tabulate our cases, we find that an excessively small proportion are in sites accessible to direct examination. Divided on this basis we have the results presented in Table 3.

These figures indicate that, as is to be expected, few of the errors are made with external tumors.

TABLE 1.—DIAGNOSIS OF NEOPLASMS

	Malignant Neoplasms		Not Cancer but Wrongly Diagnosed as Cancer
	Correctly Diagnosed	Not Diagnosed	
Accessible	399	43	28
Inaccessible	284	109	3
Total	683	152	31

One of the chief reasons why our figures of error are so high is the fact that they include very few cases of external cancer. In most cases of external neoplasms, the condition is so obvious that little or no effort is made to obtain permission for a necropsy. On the other hand, it is in the cases in which there has been difficulty in arriving at a correct diagnosis that

the permission is sought with the greatest zeal and most readily secured. Undoubtedly, the greatest influence in accounting for the large proportion of unrecognized internal tumors is the fact that so large a proportion of the cancer cases in American municipal hospitals occur in patients who are brought into the hospital when moribund or nearly so; wherefore adequate clinical study cannot, and often should not, be made. The error in diagnosis of internal tumors, 37.1 per cent., was almost exactly the same as that reported for inaccessible tumors in the London hospitals, 37.3 per cent.

TABLE 2.—PERCENTAGE OF CASES DIAGNOSED BEFORE AND AT NECROPSY

	Cook County Hospital	University of Chicago	Total	Percentage
Correctly diagnosed as malignant tumors*	257	110	367	67.34
Not recognized as malignant tumors	125	53	178	32.66
Total	382	163	545	100.00

* Irrespective of whether the location or the character of the malignant growth was correct.

As to the diagnosis of the more commonly occurring tumors, we have in Table 4 the figures on the Cook County Hospital material, which is selected as being more nearly homogeneous as to clinical conditions than the material from my own necropsies.

This table shows that few tumors of the uterus or rectum escape recognition, as might be expected. Most of the errors here were in patients dying of some complicating condition and in a state of dissolution too advanced for thorough examination. However, it is to be admitted that there were some inexcusable errors made through neglect. For example, one patient was studied most carefully by two excellent internists because of peculiar physical findings in the chest, which led to a debate as to whether the lesion might not be a pulmonary syphilis. Their examination apparently was limited to the chest, for they accepted the junior intern's note on the history that the vaginal examination was negative. There was, however, an ulcerating carcinoma of the cervix with extensive metastasis in the right pleura.

Carcinomas of the stomach, esophagus and prostate were usually recognized, although about one third were not correctly diagnosed. As might be expected, primary carcinoma of the liver and lung most often escaped recognition. Our figures in Table 5 show that, at least in the Cook County Hospital material, primary lung and liver carcinomas are far from uncommon. Primary pancreatic tumors were not often correctly diagnosed, although the malignancy was frequently recognized but not correctly located. The same is true for primary carcinoma of the bile tracts. The low figure for the diagnosis of the brain tumors undoubtedly depends on the fact that in many cases the clinical manifestations were those of a complicating hemorrhage into the brain substance, while other patients were brought into the hospital dying in coma, with no available history.

TABLE 3.—DIAGNOSIS OF EXTERNAL* AND INTERNAL TUMORS

	Cook County Hospital	University of Chicago	Total	Percentage
External tumors correctly diagnosed	61	21	82	89
External tumors not recognized	5	5	10	11
Internal tumors correctly diagnosed	196	89	285	62.9
Internal tumors not recognized	120	48	168	37.1

* Under external tumors are included carcinomas of the skin, mouth, tongue, pharynx, breast, uterus and penis, and sarcomas of the extremities or superficial structures; but not carcinomas of the rectum, bladder or larynx.

The sort of errors in diagnosis varied, of course, with the site of each tumor. Thus, in carcinoma of the stomach, the errors were extremely varied; of the thirty-six errors, the diagnosis was heart disease (usually myocarditis) in six; and cirrhosis, pulmonary tuberculosis, carcinoma of the esophagus and carcinoma of the colon in four each. In carcinoma of the esophagus, among fifteen errors, pulmonary tuberculosis appears four times, while aneurysm was the mistake but once. Carcinoma of the pancreas, diagnosed correctly in but four of twenty-two cases, was miscalled carcinoma of the stomach six times, cirrhosis four times, and carcinoma of the liver three times. Carcinoma of the bile tracts, correctly diagnosed but four times in fifteen cases, was called carcinoma of the stomach in

five cases, and the diagnosis of gallstones was made but once. Obstructive jaundice was the diagnosis in three cases. Primary carcinoma of the liver was entered correctly as liver neoplasm in but one case in ten, cirrhosis being the diagnosis in five cases. Primary lung tumors, recognized in but one of eleven cases, and interpreted as intrathoracic tumor in two others,

TABLE 4.—DIAGNOSIS OF MORE COMMONLY OCCURRING TUMORS (COOK COUNTY HOSPITAL)

Carcinoma of:	Total	Correct	Malignancy Correct, but Site Incorrect	Diagnosed Nonmalignant or No Diagnosis	Correct, per Cent.	Correct as to Malignancy, per Cent.
Stomach	103	67	11	25	65	76
Esophagus	41	26	3	12	63	70
Pancreas	22	4	9	9	18	59
Bile tracts	15	4	5	6	27	60
Liver	10	1	2	7	10	30
Colon	17	8	1	8	47	53
Rectum	14	11	1	2	80	86
Bladder	18	8	3	7	44	61
Prostate	8	5	1	2	63	75
Uterus	21	17	1	3	81	86
Lung	11	1	2	8	9	27
Intracranial tumors	23	9	0	14	39	39
Renal tumors	9	4	2	3	44	67

was diagnosed pulmonary tuberculosis in four cases and pneumonia in two. The intracranial tumors, correctly diagnosed nine times in twenty-three cases, were interpreted as cerebral thrombosis and as epidemic (lethargic) encephalitis three times each, and twice each as cerebral syphilis and paresis.

Taking all the tumors of whatever location, we find the following to have been the commonest erroneous diagnoses: cirrhosis, twenty-one; pulmonary tuberculosis, eighteen; heart disease, usually myocarditis, eleven; pneumonia, seven; nephritis, six; jaundice, six; paresis, six; generalized tuberculosis, five. Naturally, in many of the advanced cases coming into the hospital, the terminal condition was recognized without the responsible malignancy being found, and so we have such diagnoses as intestinal obstruction, peritonitis, anemia, septicemia, dysentery, diabetes (one pancreatic cancer, one brain tumor), and abscess, as well as pneumonia, in several cases.

A study of the distribution of all the tumors (Table 5) reveals several significant facts. First is the great predominance of tumors of the alimentary system,

which constitute more than half of all the cases. The fact that the character of the cancer cases seen in a large public hospital is not a fair representation of the character of cancer in the community at large is indicated by the relative infrequency of the chief external cancers, i. e., the skin, six; tongue, seven; mouth, lips and pharynx, twelve; uterus, twenty-seven, and breast, sixteen. If our statistics had been compiled from the admission records of a large surgical hospital, we should have had a totally different picture, with these very sorts of operable tumors predominating over cancer of the stomach, esophagus and pancreas. In our material, we find the supposedly rare primary cancers of the liver, fifteen, and lung, seventeen, ranking with cancers of the mammary gland, sixteen, while cancers of the pancreas, thirty-two, and intracranial tumors, thirty-three, exceed the uterine cancers, twenty-seven, which barely outnumber the respiratory tract tumors, twenty-five. Despite the obviously misleading character of such selected material as these hospital statistics furnish, we find medical literature full of erroneous statements based on just such statistics.

There were fifty sarcomas among 545 malignant neoplasms, or about 9 per cent. of all. This figure is rather lower than that given in most tables of cancer statistics. Probably there is some exclusion of sarcomas of the extremities from our necropsy material for the same reason that we find external carcinomas scantily represented. Another factor is probably a more critical acceptance of the diagnosis of sarcoma than was once general. It is being appreciated more and more that not all round and spindle cell tumors are sarcomas, and that carcinomas are much more often mistakenly called sarcomas than the reverse. We have not included any of the testicle tumors among the sarcomas, where most of them are usually placed in classification, for we are convinced that they usually represent tumors of specialized cells of mesodermal origin, and that they are rarely true sarcomas.

In all these necropsies, there was but one instance of a definitely benign tumor causing death. In this case, which is not included in our tables, intestinal obstruction resulted from uterine fibroids.

There were four instances of coexistence of two apparently independent primary malignant tumors in the same subject: (1) carcinoma of the prostate coexist-

TABLE 5.—DISTRIBUTION OF TUMORS

Site and Type	Cook County Hospital	University of Chicago	Total
Alimentary canal	232	85	317
Carcinoma of:			
Stomach	103	39	142
Esophagus	41	9	50
Pancreas	22	10	32
Colon	16	3	19
Rectum	14	4	18
Small intestine	—	1	1
Ampulla of Vater	1	—	1
Bile tract	15	5	20
Liver	10	5	15
Mouth, lips and pharynx	6	6	12
Tongue	4	3	7
Genito-urinary tract	85	34	119
Carcinoma of:			
Uterus	21	6	27
Vagina	1	—	1
Mammary gland	11	5	16
Ovary	6	—	6
Testicle	7	3	10
Prostate	8	4	12
Bladder	18	5	23
Penis	2	1	3
Ureter	—	1	1
Chorio-epithelioma	1	1	2
Kidney—			
Carcinoma	4	2	6
Sarcoma	1	—	1
Hypernephroma	5	6	11
Respiratory tract	16	9	25
Carcinoma of:			
Lung and bronchi	12	5	17
Larynx	4	1	5
Trachea	—	1	1
Endothelioma of pleura	—	2	2
Cerebral tumors	24	9	33
Glioma	20	5	25
Sarcoma	2	2	4
Endothelioma	1	2	3
Carcinoma, choroid plexus	1	—	1
Ductless glands	10	5	15
Thyroid	5	3	8
Hypophysis	3	—	3
Suprarenal	2	2	4
Sarcomas	57	21	78
Total, all sites	37	13	50
Melanosarcoma	5	2	7
Retroperitoneal	3	3	6
Mediastinal	7	—	7
Extremities	5	3	8
Endothelioma	1	4	5
Brain	1	2	3
Pleura	0	2	2
Miscellaneous	11	17	28
Carcinoma of skin	4	2	6
Carcinoma of antrum	—	1	1
Neuroblastoma	2	1	3
Chorio-epithelioma	1	1	2
Multiple myeloma	1	4	5
Carcinoma, primary site not located	2	4	6
Multiple tumors, cases	1	3	4
Chloroma	—	1	1

ing with a carcinomatous ulcer in the pylorus, which perforated into the peritoneum; (2) glioma cerebri in a man with a small fibrosarcoma in the skin of the thigh; (3) scirrhous carcinoma of the head of the pancreas with a typical ulcerating carcinoma of the stomach, and (4) giant cell sarcoma of the jaw with carcinoma of the prostate.

This study shows emphatically the lack of value of all recorded vital statistics on cancer. When we find diagnostic errors ranging from 25 to 40 per cent. in patients who have been examined in modern hospitals in Germany, England and America, with the advantages of exploratory operations, roentgen rays and laboratory studies under the most competent medical men in the community, it is certain that the diagnostic errors made throughout the country at large must be even greater. To be sure, to the large charity hospitals come an excessive proportion of patients too near death for careful study, and there come to necropsy an undue proportion of cases that are difficult of diagnosis. On the other hand, it is quite certain that a large proportion of persons who have internal cancers are not seen by physicians much earlier than are these hospital patients. It is also certain that, under the conditions obtaining in general practice, the diagnostic effort and ability will average much less than in the large hospitals.

These necropsy statistics show convincingly that we have at the present time no reliable statistics relative to the frequency of cancer as a cause of death. The futility of attempting to learn anything concerning heredity from such statistics is even more glaringly apparent. A single error in diagnosis may lead to entirely erroneous conclusions in the study of the influence of heredity on cancer in a given family. Such accuracy is obviously not present in any existing study of human heredity in respect to cancer. We shall have no exact and very little useful information concerning cancer statistics until a very much larger proportion of vital statistics depends on postmortem examination than is now the case.

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THE INFLUENCE OF HEREDITY ON THE OCCURRENCE OF CANCER*

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Until recently it has not been possible to speak with assurance concerning the influence of heredity on the occurrence of cancer; the evidence at hand has been of such an unsatisfactory character that the validity of whatever conclusions were drawn was always open to question. Numerous attempts to secure information by studying general mortality statistics, hospital populations or isolated clinical observations have led to contradictory results, as they were bound to do from the inherent errors in the data obtained from such sources. Furthermore, until within a comparatively short time, we had no definite knowledge of the principles of heredity itself, and this ignorance foredoomed to futility any speculations on the subject.

It so happens that the year 1900 saw the rebirth of two unrelated discoveries which, together, have led to an entirely new outlook on the problem, for they placed for the first time on an experimental basis the investigation of both heredity and cancer. In this year, independently, three botanists (de Vries, Correns and Tschermak) reported the rediscovery of the fundamental principles of heredity which the Austrian monk Gregor Mendel had worked out so accurately and described in 1866.

The same year witnessed the work by Leo Loeb in Chicago and by Jensen in Copenhagen, demonstrating that sarcomas in rats and carcinomas in mice can be inoculated into other animals of the same species for an indefinite number of generations—Jensen's mouse carcinoma is still being transplanted in many laboratories. The same fact had been demonstrated in 1889 by Hanau, and in 1891 by Morau, but, like Mendel's

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discovery, its significance had not been appreciated at the time and it had not been followed up. Although we have since learned that the information to be obtained from transplanted tumors is limited, especially in respect to the influence of heredity, these observations placed cancer research on an experimental basis, and the recent rapid progress in our knowledge of cancer has been largely due to the stimulus to investigation given by tumor transplantation work.

During the twenty-two years that have passed since these rediscoveries were made, genetics has become one of the most active fields of biologic research. Experimental cancer research has likewise been one of the most attractive subjects of pathology, although perhaps somewhat obscured by the contemporaneous development of the more hopeful science of immunology. In view of the relatively short period covered by these investigations, and the difficulties inherent in the problems themselves, it is not surprising that we are only just now beginning to secure evidence concerning the relation of heredity to cancer. In the following paragraphs I shall endeavor to present, as concisely as possible, the evidence that we now have concerning the influence of heredity on the occurrence of cancer.

A. *EVIDENCE FURNISHED BY HUMAN PATHOLOGY*

Human material has furnished evidence that may be classified in two chief groups: (1) statistical investigations and (2) cancer families.

STATISTICAL EVIDENCE

Statistical evidence may be dismissed with the statement that, in the question of human cancer heredity, *all existing statistical evidence is valueless for any exact information on the subject,* and it must remain so until such time as we have necropsy records on all persons dying in several generations. When we consider how few persons have knowledge of a physician's diagnosis of the fatal illnesses of even their grandparents, to say nothing of their great uncles and aunts and of preceding generations, we at once recognize the limitations of clinical histories in casting light on the relation of human heredity to cancer. And even

if we did have such records of clinical diagnosis we should be no better off, for the error in such diagnoses is far too large to permit us to use them.

We find that, even in large modern hospitals, necropsies show an error in clinical diagnosis in respect to cancer of anywhere from 20 to 50 per cent., depending on how large a proportion of the cases are of malignancy in the internal organs.[1] With such a great error in the diagnoses in large hospitals, where every modern diagnostic resource is available, what must the error be in the clinical diagnoses made on private patients under the varied and unfavorable conditions that often obtain even in present days, to say nothing of the diagnoses in previous generations before the days of microscopy or modern methods of diagnosis?

In considering the relationship of heredity to cancer, even a single error in diagnosis might totally destroy the value of an entire family history, making it appear that cancer was present in a branch of a family when it was not, or that it had failed to appear in a certain branch in which it actually did exist. Therefore, the records that exist now, or that may be obtained in the future, can be of no value whatsoever for this purpose until they all contain a complete postmortem examination with microscopic control.

With these essential facts before us, we must dismiss the numerous discussions in the literature based on mass statistics. By so doing we lose nothing, for the conclusions are as contradictory as possible. On the one hand we have the much-quoted and carefully compiled statistics of an expert statistician, Karl Pearson, using an absolutely worthless material, the histories of patients in a large charity hospital as to the incidence of cancer in their known relations. These failed to show any evidently greater proportion of cancerous relations in cancer patients than in patients with other diseases. Any medical man who has questioned the patients of such a hospital knows how absolutely worthless is the information that the patients can give in respect to family history. Most of them do not know how many and which of their relatives are living, to say nothing of the causes of death. Thus, in a

1. Wells, H. G.: Relation of Clinical to Necropsy Diagnosis in Cancer, and Value of Existing Cancer Statistics, J. A. M. A. **80**: 737 (March 17) 1923.

discussion of the relation of heredity to cancer, Bashford [2] reports that in the histories of 2,932 cancer patients, in respect to familial occurrence of cancer, "no reliable statement was obtainable for 2,263." That is to say, in less than one fourth of the cases was there even a layman's knowledge of cancer in the family, although in the 4,526 parents there undoubtedly were not far from four or five hundred cases of cancer. To use refined statistical methods on such material is as useless as some of the attempts that are made to use the extremely exact methods of physical chemistry in interpretating biologic processes that even in controlled conditions show variations of 50 per cent.

It is only fair to Pearson to quote his own conclusion:

> The data do seem to justify further inquiry and to suggest to my mind that possibly the tendency to cancer does not run strongly in families. I admit that I should accept this only with reluctance, for general health, I find from my own investigations, is inherited exactly as any physical character like shape of the head, and length of life, is inherited also. It is difficult to conceive that longevity and general health can be inherited if the tendencies to particular diseases do not largely run in families. Cancer may be an exception, of course, and certainly the above results, without being convincing, do call upon us to pause and ask for further inquiry.

From this it is seen that Pearson was much more conservative in his deductions than most of the writers who have quoted his carefully analyzed statistics as weighty evidence that there is no hereditary influence in cancer.

On the other hand, we may find as many sets of statistics as we wish, which, like those of Williams,[3] show that a very considerable number of the relatives of cancer patients have had cancer; but they are not usually so controlled as to be of any value, even if such mass statistics could mean anything. About the only thing we can deduce from them is that most of the published statistics indicate that usually a larger proportion of the relatives of cancerous subjects have been cancerous than of the relatives of noncancerous patients. Few investigators, like Pearson, find the proportion of cancerous relatives the same in the two

2. Bashford: Proc. Roy. Soc. Med. **2**, Part 1, p. 63, 1909.
3. Williams: Natural History of Cancer, New York, William Wood & Co., 1908, p. 367.

groups, and still fewer, if any, find more cancer among the relatives of the noncancerous subjects.

Of material of this sort, perhaps the best is that considered by Little.[4] This is contained in the family history records of the Eugenics Record Office of the Carnegie Institution, which have been furnished by persons of intelligence, conscientiously endeavoring to provide accurate family histories for scientific purposes. Analysis of these records shows that cancer occurs much more frequently among the descendants of cancerous parents, or in persons with cancerous relatives, than is to be expected from the general cancer mortality figures. However, we have here the usual defect in that the occurrence or absence of cancer is based on only the layman's belief concerning the cause of death of relatives, and the vital statistics used for comparison are of no more accuracy than any statistics not based on necropsy records, and probably give too low a cancer rate.

CANCER FAMILIES

Much attention has been given to the occurrence of families in which a strikingly large proportion of members are cancerous. The existence of such families cannot be denied. Some of these families have become classical in cancer literature. Such is the Bonaparte family; for Napoleon I, his father, his brother Lucien, and two of his sisters, Pauline and Caroline, all were believed to have died of cancer of the stomach. One of the most remarkable is the family of Madame Z, reported by Broca in 1866, and mentioned in virtually every discussion of heredity in cancer since that time. Many more such families might be described. The chief trouble with most of these reports is that they do not include all the noncancerous members of the family, and hence we cannot always be sure that the incidence is really so exceptional. Furthermore, they have, of course, the defect of depending only on family traditions and belief as to the cause of death.

In family records with a high incidence of cancer, no matter how high the proportion is, the possibility that the heaping up of cases in these families may

4. Little: Eugenics, Genetics and the Family, **1**: 186, 1923.

depend on chance cannot be evaded. Granted that of the entire population past 40, about 10 per cent. will have cancer, the laws of probability would determine the occurrence of occasional families in which a high proportion of cancer cases would occur if heredity had no influence at all. Bashford [5] gives the accompanying table showing how large a number of cases of cancer are to be expected on this basis, independent of any other influence than that of chance.

Likewise, there should be fortunate families that escape cancer through pure chance. As far as I can learn, no one has sought families that show an immunity to cancer, although such family records should be more attractive than the other kind.

Occurrence of Cancer in Families, According to the Laws of Probability *

Number of Cancer Deaths in Family	Per Hundred Families of		
	6 Members, viz., 3 Men, 3 Women	8 Members, viz., 4 Men, 4 Women	10 Members, viz., 5 Men, 5 Women
None	51	41	33
One	36	39	39
Two	11	16	20
Three or more	2	4	8
	100	100	100

* Including only persons living thirty-five years or more.

But when we find families that have many cases of a certain sort of tumor which is not common, or a tendency to frequent location of a certain tumor in a certain place, the law of probability becomes inadequate. We have just such records.

Undoubtedly the retinal glioma families afford the most striking examples of such unquestionable heredity influence. Glioma of the retina is a rare neoplasm, which is striking in that it often occurs in infants or even at birth. Its familial occurrence is equally striking, and we have numerous records of such families as the following:

1. Newton [6] has reported a family of sixteen children, of which ten had died of retinal glioma, the

5. Bashford: Proc. Roy. Soc., Med. Sec. **2**: 67, 1909
6. Newton: Australian M. Gaz., May, 1902, p. 236.

disease being bilateral in seven cases. Two of the others died in infancy, and four were alive and well. Both parents were free from tumor, but a brother of the father was believed to have died of the same disease.

2. Wilson [7] reported a family in which eight children had retinal glioma.

Fig. 1.—Glioma of retina (patients of Dr. L. Comas of Santiago). The boy is the only survivor of five children with retinal glioma, both eyes having been enucleated. The sister shown in this picture died shortly after the photograph was made.

3. Purtscher [8] has described a glioma family: A man who died of sarcoma of the arm had eleven children; two of six boys died of glioma of the retina (at 9 and 3 years); a daughter had bilateral glioma (verified by Fuchs), which retrogressed spontaneously. Her only child, a boy, died of glioma. Two children

7. Wilson: Tr. Path. Soc. Dublin, 1871-1874, p. 138.
8. Purtscher, reported by Peller: Wien. klin. Wchnschr. 35: 121 (Feb. 9) 1922.

of the members of the family, who remained tumor free themselves, developed glioma; one died and in the other the tumor retrogressed during ten years while under observation in the clinic of Fuchs.

4. Comas [9] has described a striking instance of familial glioma: Among eleven brothers and sisters, five have had glioma of the retina. Of the five, three sisters and two brothers, only one brother is alive; he is now 12 years old and enjoys good health, although blind. Both his eyes were enucleated, the left when he was 2 years old and the right six months afterward (Fig. 1). Recently, Dr. Comas writes me, the father has died from gastric cancer. The mother is in good health, and the other children, one of them 16 years old, are also in good health. The illustration (published with the permission of Dr. Comas) shows the mother, the blind brother, and one of the sisters who died shortly after this picture was taken.

It may be suggested, not without justification, that retinal glioma is not a typical and unquestionably malignant neoplasm, for it is often bilateral, does not commonly produce remote metastases, and may even retrogress spontaneously (witness one of Purtscher's cases). I therefore add some other examples of familial neoplasms.

Silcock [10] describes the following instance: A mother and daughter each suffered from melanosarcoma of the choroid of the left eye, each identified microscopically. The mother's father and each of her twin sisters had had an eye extirpated because of disease, but that this was melanosarcoma in all three cases is not known.

Williams [11] has collected a large number of family groups in which several members of the same family have died of similar or identical sorts of neoplasms, some of the instances being very striking, as are also the families described by Bashford,[12] Pel,[13] Peiser,[14] Watkins,[15] Richards,[16] and Oidtman.[17]

9. Comas, L.: Five Cases of Glioma of the Retina in One Family, J. A. M. A. **75**: 1664 (Dec. 11) 1920.
10. Silcock: Brit. M. J. **1**: 1079, 1892.
11. Williams: Natural History of Cancer, p. 356.
12. Bashford: Proc. Roy. Soc. Med. **2**: 71, 1909.
13. Pel: Berl. klin. Wchnschr. **52**: 288, 1915.
14. Peiser: Med. Klin. **11**: 193, 1915.
15. Watkins: Brit. M. J. **1**: 190, 1904.
16. Richards, C. M.: Am. J. Roentgenol. **8**: 514 (Sept.) 1921.
17. Oidtman: Nederl. Tijdschr. v Geneesk. **1**: 2184, 1917.

As especially significant may be cited the interesting cases reported by Burkard [18] of twin sisters, aged 21 years, who developed each a fibro-adenoma of the left breast, each of the same microscopic structure, at almost the same time and in the same part of the breast. This recalls the remarkable family described by Critzmann,[19] in which all members who were *not* twins died of cancer, all the twins escaping. Such an instances must be related to Galton's family, remarkable for the number of twins; whenever single children were born they always had six fingers and six toes, but the twins always had the normal number.

Another striking instance is reported by Hedinger [20] of primary liver cancer in two sisters examined at necropsy within one week. No cancer was known in the family, but Hedinger believes it impossible that two such rare tumors could have occurred in sisters without a familial organ disposition. Another case of rare tumor in sisters is reported by Primrose,[21] namely, primary "carcinoma" or cancroid of the appendix.

Some interesting data are given by Peller [22] from his analysis of a questionnaire sent out to the entire medical profession of Austria, from whom 389 replies were received; but here the factors of selected material and uncontrolled data make the significance of the reported material of uncertain value, for it is probable that observations in which many of the members of a family are cancerous are especially often recorded because they are especially striking.

Perhaps the most reliable family records of cancer in the literature are those reported by Warthin,[23] which have the virtue of being based on histologic examination in at least many of the cases, and in being collected in a university hospital population probably with better information as to family history than is likely to be the case in large city hospitals with a foreign-born clientele. He cites with diagrams several families in which a striking history of cancer was obtained. In one, of forty-eight descendants of a cancerous grandfather, seventeen had died or been operated on for

18. Burkard: Deutsch. Ztschr. f. Chir. **169**: 166, 1922.
19. Critzmann, cited by Williams: Natural History of Cancer, p. 374.
20. Hedinger: Centralbl. f. allg. Path. **26**: 385, 1915.
21. Primrose, Alexander: Ann. Surg. **72**: 690 (Dec.) 1920.
22. Peller: Wien. klin. Wchnschr. **35**: 121, 1922.
23. Warthin, A. S.: Heredity with Reference to Carcinoma, Arch. Int. Med. **12**: 546 (Nov.) 1913.

cancer, and many of the survivors were below the usual cancer age when the paper was published. The preponderance of carcinoma of the uterus (ten cases) and of the stomach (seven cases) is striking. Another family, in which the father and mother escaped cancer although each had cancerous brothers and sisters, consisted of three, daughters, all of whom had uterine or ovarian tumors. Especially striking is a family in which a cancerous father and a cancerous mother had six children, all of whom died of cancer, as did the only grandchild; that is, of the entire family of nine members in three generations, all died of cancer, as did the paternal great grandfather.

Warthin [24] thus sums up his study:

> In the histories of cancer cases coming from the state of Michigan and examined at the pathological laboratory of the university, about 15 per cent. show a striking history of multiple family occurrence. When the difficulty of obtaining good histories is considered, this proportion is relatively high, and, on the whole, corresponds fairly closely with the percentage obtained by Williams. We must conclude, then, that a definite and marked susceptibility to carcinoma exists in certain families and family generations. This family tendency is usually most pronounced when there is a history of cancer in both paternal and maternal lines. In such families there is an especial tendency for carcinoma to appear at an earlier age than in the forebears, and in these younger individuals the cancer usually shows an increased malignancy.

Wolff,[25] in his great compilation of cancer literature, says that recent authors find that from 11 to 18 per cent. of their cancer cases show evidence of an hereditary influence. After discussing this evidence, he concludes:

> Nevertheless, we must admit that heredity may be a factor for predisposition to cancer, for it is admitted that heredity is seen in nevi, warts and other skin abnormalities which are known to be closely related to cancer formation; furthermore, heredity is understandable on the assumption that abnormalities in fetal development, which likewise are often the starting point of a cancer, may repeat themselves in several members of a family or of a generation.

He cites a report by Rüder, in which seven boys, from 5 months to 10 years of age, in one family exhib-

24. Warthin, A. S.: J. Michigan State M. S., January, 1914.
25. Wolff: Die Krebskrankheit, Jena **2**: 695, 1911.

ited epithelial "carcinoma" of the skin, in a form of papillary nodules up to bean size, while five girls of the same family showed none. The formation of these nodules was preceded by an inflammatory reaction where exposed to light. The course was slow, but nothing is said as to the outcome. The parents of these boys were healthy, but a grandfather had the same disease.

Those authors who consider that tumors arise usually or always in some embryonal rest of necessity lay much weight on heredity. Thus, Ribbert,[26] for instance, maintains that irritation cannot cause cancer unless there are already present the necessary germ cells, which, like all other anomalies of the germ plasma, are transmissible. He believes that ordinarily tumors develop spontaneously without exciting cause, solely because of the presence of anomalous predisposing germ cells. This view can scarcely be held now in the light of the newer studies on the experimental production of cancer, unless we agree to the proposition that nearly all individuals have such anomalous germ cells located in any part of the body that we choose to irritate with our cancer-exciting agent.

The only study of heredity in human cancer in which the mendelian principles of heredity are considered is that of Levin,[27] who used material collected by field workers of the Eugenics Record Office at Cold Spring Harbor. Unfortunately, these field workers were not physicians, and the data that they collected must have been limited to what the members of the investigated families knew or believed concerning the ailments of their relatives and ancestors. There were two "fairly complete" and three "fragmentary" records available, from a study of which this conclusion is reached:

> The incidence of cancer in these families is not greater numerically than would be found among the population of the community as a whole. But there are several points in the analysis which indicate that cancer may be influenced by heredity. A cancerous fraternity usually shows in a previous generation a cancerous member either on the maternal or paternal side or both. In other words, a cancerous fraternity

26. Ribbert: Deutsch. med. Wchnschr. **45**: 1265, 1919.
27. Levin: Ztschr. f. Krebsforsch. **11**: 547, 1912.

is usually derived from the union of two germ plasms, each of which is characterized by the presence of germ cells that are nonresitant to cancer.

It was also noted that there is a distinct family susceptibility of organs; in one family all the women with cancer had uterine involvement "except one in the omentum"; in another family group the males had intestinal cancer, the females breast cancer. Analysis of the collected statistics gave Levin evidence that *resistance to cancer is a dominant character,* the absence of which creates the susceptibility to cancer.

Benign tumors, and especially various cutaneous growths that are essentially benign neoplasms, often exhibit a striking and undeniable hereditary occurrence. Several such records of dermatologic heredity are collected by Meirowsky and Bruck.[28] Hereditary telangiectasis is also well known, the literature having been reviewed by Goldstein.[29] Multiple benign cystic epithelioma is one of the growths that has a distinctly familial distribution, and these growths,[30] which usually more or less resemble carcinoma histologically, sometimes become malignant. As illustrating the familial character of these growths may be cited the case reported by Heidingsfeld,[31] in a man of 65, whose four children, like himself, exhibited the growth on the nose; his maternal uncle and aunt had a similar growth, his mother had died at 30 without having any growths, and there had been no cases on the paternal side. Probably this is the type of growth referred to by Wolff as cutaneous epithelioma.

Another form of benign neoplasms with an extremely marked hereditary character are the multiple cartilaginous exostoses. The literature of this topic has recently been reviewed by Maynard and Scott,[32] who reported the occurrence of a family with sixteen known cases in three generations, an important feature being that the mother who transmitted the condition

28. Meirowsky, E., and Bruck, W.: München. med. Wchnschr. **68:** 1048 (Aug. 19) 1921.
29. Goldstein, H. I.: Hereditary Hemorrhagic Telangiectasia with Recurring (Familial Hereditary Epistaxis, Arch. Int. Med. **27:** 102 (Jan.) 1921.
30. Orsmby: Diseases of the Skin, 1915, p. 647.
31. Heidingsfeld: J. Cutan. Dis. **26:** 18, 1908.
32. Maynard, H. H., and Scott, C. R.: Hereditary Multiple Cartilaginous Exostoses, J. A. M. A. **76:** 579 (Feb. 26) 1921.

apparently did not herself have the disease. This has been observed in other families with this disease and is of much significance in respect to the mendelian principles of transmission of inheritable characters, as will be shown later.

Von Recklinghausen's disease, or multiple neurofibromatosis with its various related and complicating conditions, has a most striking hereditary character, at least one fifth of all patients showing close relatives with the same rare disease, which is especially significant in the fact that there is a noteworthy tendency to malignant transformation. The disease appears not only often in several members of a family, but also for many generations, its character being so striking that the lay patient is usually well aware of similar conditions in his relatives and ancestors. For example, Harbitz [33] has described a family in which the disease was known for five generations, it being of significance that in one generation it was transmitted by a woman who herself was not affected, although it was present in two of her sisters. The literature on this subject has been discussed especially by Langer [34] and by Herxheimer and Roth.[35] Davenport [36] found evidence, from an analysis of 243 cases, that the hereditary factor behaves as a dominant, coming down equally well in either male or female lines, and affecting the two sexes alike. Sometimes, however, there is a failure of dominance and generations are skipped.

Possibly of some relationship to these conditions is the central nervous sclerosis associated with multiple symmetrical adenoma sebaceum, as indicated by the family reported by Berg,[37] in which the father had tuberous cerebral sclerosis associated with adenoma sebaceum and a large, mixed, embryonal type of renal tumor; the daughter with the same nervous system disease and the sebaceous adenomas had a small angiofibroma of the kidney, and the paternal grandfather died with a large renal tumor but without the nervous

33. Harbitz, Francis: Multiple Neurofibromatosis (von Recklinghausen's Disease, Arch. Int. Med. **3**: 32 (Feb.) 1909.
34. Langer: Inaug. Diss., Leipzig, 1906.
35. Herxheimer and Roth: Beitr. z. path. Anat. u. z. allg. Path (Ziegler's) **58**: 319, 1914.
36. Davenport: Proc. Nat. Acad. Sc. **4**: 213, 1918.
37. Berg: Ztschr. f. ges. Neurol. **19**: 528, 1913.

and cutaneous lesions.[38] This disease is commonly associated with renal, cardiac and retinal[39] tumors, as well as various developmental defects, which facts bring closely together the potential relationship of inherited developmental anomalies and consequent hereditary neoplasms.

B. *ANIMAL EXPERIMENTATION*

TRANSPLANTED TUMORS

In the period of active investigation of transplantable tumors in animals that followed the demonstration by Leo Loeb and by Jensen that series inoculation of rat and mouse tumors is readily carried out, there was naturally not a little study of the influence of heredity on the inoculated tumors. At this time it was not so fully appreciated as it now is that an inoculated tumor is something quite different from a spontaneous tumor. A transplanted tumor differs from a spontaneous tumor fundamentally in that it is never a growth of the cells of the inoculated animal, but it is a growth of the cells descended from the mouse that furnished the original spontaneous tumor from which the transplanted growth was obtained. For example, a mouse inoculated with a strain of the Jensen carcinoma, which has been carried through myriads of generations of transplants during the twenty and more years since Jensen first started the transplantation, is growing a tumor composed of cells derived from Jensen's original tumor mouse and not from its own tissues. The mouse bearing an engrafted tumor is merely furnishing the soil on which some grafted tissue is growing, exactly as a culture tube furnishes a soil on which bacteria are growing.

That the inoculated cancer is fundamentally different from the spontaneous cancer is shown by the fact that successfully inoculated growths often disappear spontaneously, and that protection may be furnished by various procedures of immunization, phenomena which are never seen in spontaneous tumors. Furthermore, animals that are immune to tumor inoculation may

38. Hanser: Berl. klin. Wchnschr. **55**: 279, 1918.
39. Van der Hoeve: Arch. f. Ophth. **105**: 880, 1921.

develop spontaneous tumors,[40] and by painting the skin with tar, true carcinomas may be produced in mice immune to grafts of the same sort of tumors.[41]

Therefore observations on the influence of heredity on the susceptibility of animals to transplanted tumors can have no direct bearing on the question of susceptibility to spontaneous tumors, since the resistance to one bears no direct relation to the resistance against the other. The chief things of interest learned from the inoculated tumors in respect to heredity are the following:

1. Close relationship of animals is favorable for inoculation. An animal inoculated with its own spontaneous tumor is more likely to develop growths from the inoculation than any other animal of the same species;[42] its close relatives are more likely to give positive results than unrelated mice, and the likelihood of successful inoculation becomes more and more remote, the more different in origin and in character the inoculated mice are from the originator of the tumor. Growing a transplantable tumor for several generations in a particular strain of animals may enhance its virulence for that particular strain of animals and not for others. These and other related facts indicate that even with transplantable tumors there are differences in susceptibility that are connected with the heredity of the animals, a point recognized in the pioneer investigations of Morau.[43]

2. Certain strains of animals are insusceptible to tumor grafts to which other strains of the same species are susceptible. This has been frequently observed, and the differences in susceptibility may be either absolute or only quantitative. For example, Haaland[44] found that a certain tumor grew in nearly 100 per cent. of Berlin mice, in 24 per cent. of Hamburg mice, and was practically innocuous to Christiania mice. Again, Loeb has described a tumor originating in a Japanese waltzing mouse, which grew in nearly 100 per cent. of

40. Thorel: Verhandl. d. deutsch. path. Gesellsch. **12**: 60, 1908. Clunet: Recherches expérimentales sur tumeurs malignes, Paris, 1910, p. 24. Bashford, E. F.: Fourth Sc. Rep. Imp. Cancer Res. Fund, 1911, p. 19.

41. Fibiger: Act. chir. scand. **55**: 343, 1922.

42. Loeb and Leopold: J. M. Res. **17**: 299, 1908.

43. Morau: Arch. med. exper. u. anat. Path. **6**: 677, 1894.

44. Haaland, cited by Tyzzer, E. E.: J. M. Res. **21**: 519, 1909.

this sort of mice but not at all in common laboratory mice. Many other similar instances have been reported.

3. Heredity influences in a constant manner the susceptibility of a given strain of animals to inoculation with cancer. For example, Tyzzer [45] studied a carcinoma which arose in a Japanese waltzing mouse and which could be inoculated into mice of the same type with a large percentage of success, but which did not grow in certain "common" strains of mice. When Japanese waltzers were bred with these common mice the mice of the first hybrid generation were all susceptible, but the second and third hybrid generations made by cross breeding the first generation hybrids were insusceptible. The mice of the first hybrid generation, although susceptible to tumor inoculation, did not show the waltzing character of the susceptible parent strain, whereas the waltzing mice (recessives) that appeared in the second and third hybrid generation were not susceptible to inoculation with the dancing mouse tumor. Further studies carried out with numerous back crosses gave results that indicate that susceptibility to *grafted* tumors is not inherited as a single mendelizing factor, for they do not furnish a ratio characteristic of a single factor inheritance. Tyzzer and Little believe that both susceptibility and nonsusceptibility are inherited as a complex of mendelizing factors, perhaps as many as twelve or fourteen in number.[46] Susceptibility to this tumor might seem to be a dominant character, since it appears in the first hybrid generation; but its failure to appear in the subsequent hybrid generations does not conform with the behavior of a dominant unit character.

Loeb and Fleisher [47] have experimented with crosses of strains of domestic mice with differing susceptibility to transplantable cancer and obtained results not altogether in agreement with those of Tyzzer. For example, in the first hybrid generation the susceptibility to inoculation was intermediate between that of the two ancestors, and not as high as in the more susceptible. The second and third hybrid generations showed very

45. Tyzzer, E. E.: J. M. Res. **16**: 519, 1909; J. Cancer Res. **1**: 125 (April) 1916.

46. This conclusion has been reinforced by Little in a subsequent communication on the same material (J. Cancer Res. **6**: 106, 1921).

47. Loeb, Leo, and Fleischer, M. S.: Centralbl. f. Bakt. u. Parasitenk. **67**: 135, 1912.

little susceptibility, but in the fourth and fifth generations there was increased susceptibility. They agree with Tyzzer and Little in believing that, if susceptibility to these inoculated tumors is a mendelian process, it must depend on multiple factors. In later experiments,[48] using different strains of animals, they obtained entirely different results, indicating the lack of fixed principles applicable to all transplanted tumors, and all strains of animals. In one strain a pure line was extracted with a susceptibility different from that of the rest of the strain.

In rats also the transplantability of tumors has been observed to be influenced by heredity; but in Roffo's[49] experience this behaved more like a simple mendelian character than in the observation of Tyzzer and Loeb with mice. On the other hand, Morpurgo and Donati[50] were unable to demonstrate that twenty-nine offspring of rats bearing inoculated tumors were more or less susceptible to inoculation than the twenty-eight offspring of rats of the same strains that were immune to inoculation. This again disagrees with the observations of Levin and Sittenfield,[51] who found also in a relatively small number of animals (twenty) that a transplantable rat sarcoma that gave 86 per cent. of takes gave but 25 per cent. in the offspring of immune rats, a figure indicating that resistance to this transplantable tumor behaves as a dominant character. The number of animals used in these two last sets of experiments is so small, however, that the results have little if any value.

SPONTANEOUS ANIMAL TUMORS

Except for the extensive studies of Loeb and Lathrop and of Maud Slye, which will be considered later, most of the evidence as to inheritance of a tendency to spontaneous tumor development in animals is of a somewhat casual nature. However, there are several interesting observations on this point. Perhaps the oldest record of this sort appears in the classical treatise on tumors by Virchow, "Die krankhaften

48. Loeb, L., and Fleisher, M. S.: J. Cancer Res. **1**: 331 (April) 1916.

49. Roffo, A. H.: Rev. Inst. Bact. Buenos Aires **1**: 441, 1918.

50. Morpurgo, B., and Donati, A.: Munchen. med. Wchnschr. **60**: 676, 1913.

51. Levin, L., and Sittenfield, M.: Proc. New York Path. Soc. **10**: 119, 1910.

Geschwülste," published in 1864. Here, in discussing the melanosarcomas of horses, Virchow [52] says:

> Brugnone, who first seems to have described this disease of horses, although under the name of hemorrhoids, reported that a white stallion introduced this disease into the animals of a herd in Sardinia, and that his progeny developed melanosarcoma, whether male or female. Gohier has told of a report by Gallety-Latournalle that a young white stallion affected with black growths transmitted this condition to all its white progeny, while all those that were not white remained free. As a result, the disease spread itself over the entire province of Bresse and its vicinity.

Coming to more modern times and experimental methods, in 1898 we find Eberth and Spude [53] describing the occurrence of a family of mice with mammary gland tumors. In 1907, Tyzzer [54] made observations on a number of mice in respect to the occurrence of spontaneous tumors in the offspring of tumorous and nontumorous ancestors; but the breeding was not followed through generations enough to establish any basis for deduction as to how any possible hereditary influences might work. One family of mice (C), descended from a mouse with a papillary cystadenoma of the lung, exhibited three similar tumors among twenty-four offspring, none of which lived over fourteen months. Family B, consisting of twenty-nine offspring of a mouse with a lymphoma, produced one lymphoma and one lung tumor, but only seven of these twenty-nine lived to be 1 year of age. Family A continued ninety-eight offspring of a mouse with a lung cystadenoma, of which sixty-five lived more than six months, and of these twenty (32 per cent.) presented tumors, of which seventeen were tumors of the lung.

The conclusion was reached from these observations that they "indicate that one of the factors in the development of tumors is to be found in an inherited character or peculiarity." Still earlier, Leo Loeb [55] had suggested heredity as an explanation of so-called cage epidemics in laboratory animals and the occurrence of "epidemic" conjunctival carcinoma in cattle.

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52. Virchow, Rudolf: Die krankhaften Geschwülste **2**: 241, 1864.
53. Eberth and Spude: Virchows Arch. f. path. Anat. **153**: 60, 1898.
54. Tyzzer, E. E.: J. M. Res. **17**: 199, 1907; **21**: 479, 1909.
55. Loeb, Leo: Centralbl. f. Bakteriol., I, Orig. **37**: 236, 1904.

Murray[56] investigated the influence of heredity on the occurrence of spontaneous tumors by breeding together animals descended from cancerous females, or by breeding cancerous females with males descended from cancerous females, and by breeding together the offspring of such matings. Among 340 female mice so obtained, which reached an age of 6 months or over, sixty-nine, or 18.2 per cent., had some form of tumor (sixty-two being of the mammary gland), whereas in 223 females without known tumors in either their mothers or grandmothers, only twenty-three, or 8.6 per cent., had tumors. The value of this study is limited by the fact that the ancestry of these mice is known for such a short distance; but as far as it goes it indicates, says Murray, that:

> Female mice in whose ancestry cancer of the mamma has occurred not farther back than the grandmothers are distinctly more liable to develop the disease spontaneously in this organ than those in whose ancestry cancer is more remote. The increased liability is probably of the nature of a predisposition of one particular tissue or organ system to undergo cancerous transformation under the wear and tear of life. The differences between the two groups are apparent at all ages, and the age of maximum incidence does not appear to have been lowered in the predisposed group. The magnitude of the difference is such that it cannot be accounted for by the chances of random sampling.

In such a method of selection as employed by Murray, however, there exists a fundamental source of error, for selecting merely cancerous and noncancerous *individuals* it was perfectly possible for some of the cancerous mice to have been members of strains or families in which the tumor rate was low, the cancerous individuals having been exceptional members of these strains; equally well, the noncancerous individuals might have been exceptional members of strains or families in which the cancer rate was high. It is of interest to note that among the mice of immediate cancerous ancestry in Murray's experiment were eight cases of lymphoma, all occurring in the descendants of two mice with mammary gland carcinoma. Although there were but four squamous cell carcinomas, three of them occurred in sisters in a single litter. These two

56. Murray, J. A.: Fourth Sc. Rep. Imp. Cancer Res. Fund, 1911, p. 114.

last observations, together with those of Tyzzer, suggest that type and location of spontaneous tumors may be influenced by heredity; but the number of cases and character of control does not by any means establish this as a general law.

Another item bearing on this subject is given by Creighton,[57] unfortunately without sufficient details, in these words:

> I was enabled, by the kindness of Mr. F. W. Twort, to use a whole series of microscopic specimens from thirty cases of mouse-cancer, female and male, which came all from one or two cages in the following circumstances: A breed had been started two years before in the country as a pastime. Cases of tumor occurred from time to time, of which the animals died, microscopic specimens being made from each of them as they occurred. The fatalities continued, no new blood was introduced, and at length the whole of the mice, males as well as females, died of one kind of cancer or another, so that the family was literally exterminated in the space of two years.

A much more extensive series of observations than any of the foregoing has been reported by Leo Loeb and Abbie Lathrop,[58] based on the existence in the stock of mice bred by Miss Lathrop, for commercial purposes, of strains in which subcutaneous (presumably chiefly mammary gland) tumors were found with almost constant frequency, the rate varying widely in different strains. Only females over 6 months of age were considered. Unfortunately, these tumors were not all examined microscopically, nor was there systematic necropsy investigation of mice in which no external cancer occurred; but it is understood that the figures are based solely on the presence or absence of visible or palpable mammary gland tumors, diagnosed often only by observation of the gross appearances. If so, this fact needs to be kept in mind in analyzing the results, which should be considered as applying exclusively to macroscopically recognizable mammary gland tumors. Just how much of an error this will introduce into the figures is uncertain, and we cannot be sure that the proportion of error will be approximately the same in different series, since it would be possible for one

57. Creighton, Charles: Some Conclusions on Cancer, London, Williams and Norgate, 1920, p. 27.

58. Loeb, Leo; and Lathrop, Abbie: J. Exper. Med. **22**: 646, 713, 1915; **28**: 475 (Oct.) 1918; J. Cancer Res. **4**: 137 (April) 1919; **6**: 197, 1921.

series to have a considerable proportion of internal neoplasms not present in another strain. Loeb is of the opinion, however, that omitted internal tumors could not have been sufficiently numerous to modify their statistics seriously.

The main points developed by Loeb and Lathrop are these: Heredity undoubtedly is an important factor in determining the incidence of cancer, for strains can be established which run an almost constant proportion of subcutaneous cancer for several generations. These rates may be as high as from 58 to 65 per cent. They also found that a certain relationship exists between tumor frequency and tumor age, the latter seeming to be as characteristic for a certain strain as the tumor rate, or perhaps more so. When strains known to differ in their tumor rates were crossed, it was found that the hybrids might show tumor rates corresponding to those of either parent, or intermediate between the rates of the parents. Even when the two parents have a similar rate, the offispring may have a different rate, although in such a hybrid cross the rate is usually similar to that of the parents.

The relations between tumor age and tumor rate, previously mentioned, are interpreted by Loeb and Lathrop as best explained "if we assume that the hereditarily transmitted constitution, so far as it represents the tendency of the organism to develop tumors, depends on the cooperation of multiple factors. These multiple factors determine the intensity in the tendency to tumor development in a certain individual. In general, the greater is this intensity, the earlier do the tumors appear, and the greater is the probability that in related individuals there exists likewise a tendency to the development of tumors. It is furthermore probable that in addition to the general factors determining the intensity toward the development of cancer, there exist factors which determine specifically the tumor age in certain individuals and strains."

A most extensive and carefully controlled investigation on the influence of heredity in the incidence of tumors in mice has been conducted by Maud Slye[59] under the auspices of the Otho S. A. Sprague Memorial

59. A review with full bibliography is given by Slye in the Journal of Cancer Research 7: 1-67. 1923.

Institute. This work has continued for more than twelve years, being the continuation of a study in genetics begun under the direction of Prof. C. O. Whitman, and has been carried out with a stock of mice of known ancestry for many generations. These mice are all the descendants of a limited and carefully selected stock, bred together according to definite plans designed to give evidence as to the influence of heredity on the incidence of spontaneous tumors in mice, and hence including strains of highly cancerous ancestry and strains with ancestry free from cancer. They represent strains in which cancer is very common, strains in which it never occurs, and strains of intermediate character. It must also be emphasized that not one of these mice has been subjected to any artificial influences that might modify its life. In no case is a spontaneous tumor used for inoculation, or operated on, and no mouse born in this laboratory is ever used for any experimental work whatever. From the moment of its birth, every effort is directed to the one object of permitting each mouse to reach a maximum age. Long experience and great care have made it possible to limit to a large extent the epidemic infections that constantly threaten such large colonies of mice under even the best of conditions. Up to the present time more than 40,000 mice have been examined postmortem, and all lesions that might possibly be cancer, or concerning which there was any doubt whatever, have been examined microscopically by Miss Harriet Holmes and myself. For example, every pneumonic lung is sectioned, no matter how obvious the pneumonia, since the inflammatory lesion might possibly conceal a lung tumor. All told, there have been at least 5,000 spontaneous tumors, mostly malignant, observed in this stock; and as all the mice, whether evidently cancerous or not, have been submitted to necropsy, the material is adequate in amount and quality to furnish evidence against which the question of chance or inadequate controls cannot be raised. Were it not that every dead mouse is thus thoroughly investigated, and that the average age at death is, for a mouse community, very high, there would not be nearly so much cancer material.

Out of this work many facts have come and much has been learned concerning the influence of heredity

not only on the occurrence of cancer, but also on its behavior. Some of the outstanding results are the following:

1. *Cancer in mice appears in most of the forms seen in man, and in far greater variety than had previously been supposed.* Since most of the mouse tumors that have been studied elsewhere were those observed by animal breeders, and generally in comparatively young mice, almost the only mouse tumors that were known for some time were mammary gland tumors. Only when large numbers of mice were kept to a maximum age, permitted to die a natural death, and then studied carefully by necropsy, did the great variety and abundance of mouse tumors outside the mammary gland became known. Although mammary gland carcinoma is by far the commonest growth, nevertheless not a few other growths are found. Thus, in the first 6,000 mice were 160 with primary tumors of the lung, chiefly papillary adenomas and carcinomas; in the first 10,000 mice there were twenty-eight primary liver tumors, this number having been greatly increased since that time; in 12,000 necropsies, no less than eighty-seven unquestionable sarcomas were found; in 19,000 mice there were twenty-eight cases of tumor of the testis; in 28,000 mice there were seventy-one cases of squamous carcinoma of the skin or mouth, fifteen cases of typical basal cell carcinoma of the head, four squamous cell carcinomas in the stomach, and several others in different locations; in 22,000 mice there were forty-four with ovarian tumors, mostly solid papillary adenomas; in 33,000 mice there were sixteen primary renal and four suprarenal tumors; in 39,000 necropsies were found twenty-two uterine tumors, eleven of which were leiomyomas, and no unquestionable carcinomas. Not a few other tumors have been observed in these mice, which will be reported later. The importance of the demonstration of this abundant variety of tumors in mice lies in the fact that it adds greatly to the existing evidence of a fundamental similarity in neoplastic disease of mice and that in man. Nearly all tumors found behave in much the same way, occur at a corresponding period of life, in response to similar conditions, and present exactly the same histologic structure as similar tumors in man. The chief difference lies in distribu-

tion, the mammary gland carcinomas being by far the most frequent, whereas carcinomas of the stomach and uterus are almost unknown in mice, the same being true for nearly all animals except man.

2. *The tendency to develop cancer, or the capacity to resist cancer, is unquestionably influenced by heredity.* Strains have been established in which, among many hundreds of individuals, through as long a period of observation as twenty-five or thirty generations, not a single case of tumor growth has been seen. Also, strains have been established in which the occurrence of cancer is so common that it becomes the sole cause of the natural death of the animals. Since every mouse that dies of anything except senility might possibly have developed cancer had it lived out its maximum possible span of life, it is difficult to secure 100 per cent. cancer strains; yet families have frequently been obtianed in which all the deaths for one or more generations have been from cancer, and strains with figures approaching 100 per cent. tumor incidence for several generations have been obtained.

Since cancer is a disease developing late in life, what is transmitted is merely the tendency, or resistance to the tendency, to acquire cancer, never the disease itself. But, of course, it is true of all characters that only the tendency to them is inherited, since in the fertilized ovum which carries the tendency none of the characters in which it is manifested have yet appeared; some of the characters appear in fetal life, some not until after birth.

3. *The resistance to cancer in these mice behaves in breeding, in Slye's experience, like a typical mendelian dominant character. The susceptibility to cancer behaves as a mendelian recessive.* When a cancer mouse, derived from the crossing of cancer mice is crossed with a mouse free from cancer and derived from ancestors that never have shown cancer for many generations, the *resulting hybrids of the first generation never show cancer.* If such hybrids are bred together or with other hybrids of similar ancestry, cancer will appear in the offspring in mendelian proportions, and strains of (1) pure cancer mice, (2) pure cancer resistant strains, and (3) heterozygous strains can be extracted, exactly as with any other inheritable "unit character." To quote from Slye:

Cancer and noncancer tendencies segregate out and are transmitted as such. They are therefore unit characters. A specificity of tissue type in specific organs from ancestor to offspring segregates out and is transmitted as such. It is therefore a unit character. Since these things are unit characters, it is possible to manipulate them by selective breeding and thereby to implant them indelibly in any species, *or to eliminate them permanently and completely from any species.* Cancer and noncancer behave like the absence and presence, respectively, of a mechanism fitted to control proliferation and differentiation in regenerative processes; and an animal either has this mechanism or lacks it, no matter to what species he may belong.

This fact has been observed so many times and with such constancy that Slye feels certain that her work establishes her conclusions beyond any doubt. It is, of course, quite to be expected that susceptibility to cancer should be a recessive character; for, if susceptibility were dominant, cancer would be far more prevalent than it is. The usual statement that a deleterious dominant factor eliminates itself by destroying the species in which it occurs does not hold for cancer, since this disease does not usually manifest itself until after the reproductive period is almost or entirely completed.

4. *Not only the incidence of cancer is influenced by heredity, but also its site and its character.* For example, in certain strains, sarcoma is very common; in others it is seen rarely or never. In some strains one seldom sees any form of malignancy except mammary gland cancer. Miss Slye has developed one strain of mice whose inbred and hybrid derivatives have yielded more than a hundred primary liver tumors, although in all the other mice examined postmortem in her laboratory not a single liver tumor has been found, and only two other cases have been reported from the thousands of mice examined in other laboratories. Another strain has yielded a considerable number of tumors of the testis, although not a single case has ever been reported from other laboratories. A similar tendency for other tumors to appear chiefly or exclusively in certain tissues in certain strains has been observed by Slye. It may be recalled that Loeb reports that the tumors of the Lathrop stock are mostly

mammary gland carcinomas, whereas Tyzzer [60] had a remarkably high proportion of lung tumors and a low proportion of mammary gland tumors among his cases of spontaneous tumors, and Murray observed lymphomas in a certain strain of mice. Indeed, the difference in location of neoplasms in respect to organs in different species is of itself an hereditary character, for in the cow we find the mammary gland immune, the suprarenal and liver susceptible; in swine, the embryonal adenosarcoma of the kidney is the prevailing tumor; in rats, sarcomas are more numerous than carcinomas; in negroes, uterine leiomyomas seem to be exceptionally common.

5. *Behavior of tumors is influenced by heredity.* This statement is based on the observation that the localization of secondary tumors seems to be determined largely by heredity. Similar types of mammary gland tumors in mice have been found to produce many pulmonary metastases in some strains, and none or few in other strains. Furthermore, the strains in which secondary tumors occur frequently in the lungs are also the strains in which primary pulmonary tumors are common. A similar observation has also been made for the primary and secondary liver tumors. It may be mentioned that Wood [61] has observed in rats that in some strains a transplanted tumor produced many more instances of lung metastasis than in other strains inoculated with the same tumor.

6. *Inbreeding is not, of itself, responsible for an increased susceptibility to cancer.* I make this statement in this way because it has been suggested that Slye's high cancer rates were the result of inbreeding, a statement that is not warranted in the light of any appreciation of the principles of inheritance. Inbreeding merely concentrates existing characters, but does not produce new characters. Therefore, inbreeding of cancer-resistant strains produces cancer-resistant strains, while outbreeding of cancer-susceptible strains with other susceptible strains produced cancer susceptible strains. An inbred strain may produce 100 per cent. of cancer or 0 per cent. of cancer, depending on the character of the strains that are inbred.

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60. Tyzzer, E. E.: J. M. Res. **18**: 155, 1908.
61. Wood: J. Cancer Res. **4**: 63, 1919.

While we have no similar material to compare with Slye's, in which the complete ancestry for many generations is known, all animals are permitted to reach a maximum age, and every dead animal for from twenty to thirty-five generations has been submitted to careful postmortem study, yet such study as others have made of the influence of heredity on spontaneous cancer serves to corroborate at least the *fundamental* point—that heredity is a most important factor in determining the absence or occurrence of cancer.

I have previously referred to the evidence produced by Virchow, Tyzzer, Loeb and Murray in respect to spontaneous tumors in animals, which agrees in indicating the importance of heredity in determining the occurrence of cancer, and also to some extent in determining the site and character of the neoplasm. To this list may be added two more recent contributions which bear directly on the latter point.

A Swiss investigator, Stilling, had been carrying on transplantation experiments, using a definite strain of rabbits which he raised himself. During these experiments he found tumors in the uterus of several animals. He waited years until he had accumulated thirteen such animals and carried out a series of inoculation experiments with them. His death interrupted these experiments before he had given more than a few short communications concerning transplantable tumors before a local medical society. A pathologist, Beitzke,[62] examined the records Stilling left behind him and reports the results. Beitzke says:

> He [Stilling] bought almost no rabbits, but raised his own stock, which not only permitted him to control the relation of his experimental animals, but in a certain sense to raise a whole tumor race. Unfortunately, no mention was found in his records that he had undertaken an exhaustive investigation concerning the influence of heredity.

Stilling attributed his success to the fact that he kept his animals until they were old, the youngest tumor being in a 4 year, the oldest in a 7 year rabbit. He also laid weight on the fact that most of the cases occur in rabbits of the same strain. This contribution is especially valuable because rabbits seldom exhibit spontaneous tumors, for we can find in the literature records

62. Beitzke: Virchows Arch. f. path. Anat. **214**: 358, 1913.

of but twenty-eight cases of rabbits with tumor growth, all told,[63] exclusive of the thirteen found by Stilling in one small strain of rabbits. Therefore, the element of chance may be entirely excluded as accounting for the occurrence of Stilling's cases.

Equally true is this of the experience with rat sarcomas in the Crocker Fund Laboratory of Columbia University.[64] Here numerous experiments have been carried out with rats infected with a certain tapeworm, *Taenia crassicollis,* which passes its encysted stage in the liver. In some of the infected rats, sarcomas develop in the liver as a result of the irritation or growth stimulation. It has been found that different strains of rats differ greatly in their tendency to develop sarcomas in response to this common stimulation of the liver tissues, some giving a high percentage and some a low percentage of positive results. At the 1923 meeting of the American Association for Cancer Research, Wood reported that when rats that developed sarcoma were bred together, much higher rates were obtained in the offspring, such families sometimes giving 100 per cent. of positive results.

Another piece of work which may bear on the subject of heredity and tumor formation is that of Stark,[65] on the lethal neoplastic process discovered by Bridges in a fruit fly, *Drosophila melanogaster* (amelophila). This appears in the form of black granules in the bodies of the larvae, affecting males only, although not attacking sex structures, and causing death of all the affected larvae. The growth consists of a solid mass of large cells, producing much pigment which resembles melanin. Unfortunately, we know so little about the pathologic anatomy of insects that it is not possible to prove that these growths are true malignant neoplasms identical with mammalian cancer; but this much is in favor of such a conclusion: (1) the tumors consist of atypical cellular growths; (2) they invariably kill the organism they attack; (3) bacteria cannot be cultivated from them; (4) sterile eggs raised under aseptic conditions may produce larvae

63. Slye, Maud; Holmes, H. F., and Wells, H. G.: Cancer Res., to be published.
64. Bullock and Curtis: Proc. New York Path. Soc. **20**: 149, 1920.
65. Stark, Mary B.: J. Cancer Res. **3**: 279, 1918; J. Exper. Zool. **27**: 509, 1919.

exhibiting these growths; (5) the growths may be transplanted into other larvae, and into adult flies; (6) metastases may form consisting of cells identical with those of the original growth; (7) irregular mitotic figures have been noted in rapidly growing tumors, and (8) the tumors develop in embryonic rudiments, destined to develop the adult organs during the pupa stage.

If this growth of the fruit fly larvae is a true tumor, it furnishes a remarkably clear instance of a neoplasm that occurs solely on a basis of heredity which has been worked out conclusively. In the affected strains it is found that one fourth of the larvae die, and these are all males; i. e., one half of all the males die. Therefore, this inherited lethal growth behaves in inheritance as a sex-linked recessive factor.

MECHANISM OF THE HEREDITARY INFLUENCE

In view of all the experimental evidence cited above, and the absence of any experimental evidence that contradicts it, the conclusion seems inevitable that the incidence, character, location and behavior of tumors depend to some extent, at least, on the inherited qualities of the animal and of its tissues. This being granted, the next question is, How does heredity determine susceptibility or resistance to tumor formation?

Apparently this may be answered as follows: Tumor formation is the result of stimulation of the tissues to growth, the stimuli being of various sorts and nonspecific. Some stimuli produce marked proliferative effects in proportion to the retrogressive effects, and such stimuli are particularly capable of leading to neoplastic proliferation, e. g., roentgen rays, coal tar. The same amount of stimulation does not produce equal amounts of proliferative reaction in all individuals, even when of the same species; e. g., negroes are more likely to develop excessive amounts of connective tissue growth (keloids) in response to cutaneous injuries than are white men, and some white men develop more scar tissue than others from similar wounds. Not all roentgen-ray workers develop the same degree of hyperkeratosis from the same amount of exposure, and some develop roentgen-ray cancer much sooner than others. Hence there are individual variations in both

amount and character of proliferative reaction to a common stimulus, and these variations undoubtedly rest on an hereditary basis, in part if not wholly.

Evidently, then, heredity may determine whether the proliferative reaction that follows injury assumes a neoplastic character or not, just as the Crocker Laboratory rats do or do not develop sarcoma in the liver tissues about the encysted tenia, according to their ancestry. This has been pointed out especially in connection with the study of the lung tumors in Slye's mice, which show that mice with cancer heredity react to nonspecific inflammatory conditions in the lungs more often with excessive proliferation which leads to malignancy than do mice of noncancerous ancestry. All mice of tumor age have suffered more or less from inflammatory conditions in the lungs, with proliferative reactions. In some of the mice of cancer ancestry, but only in about 10 per cent. of those involved in this study, the proliferation assumed a definitely neoplastic character. On the other hand, mice not of cancer ancestry subjected to corresponding lung injury very rarely, if ever, reacted with proliferation to a degree even suggesting tumor formation. Nor is this all of the story. Since this study was made, we have learned more of organ specificity in cancer heredity, and we now know that if certain strains are selected it is possible to secure a breed of mice nearly all of which develop lung tumors, and others which never develop lung tumors in response to exactly similar conditions of lung injury.

A nicely comparable instance is cited by Bateson,[66] who, speaking on observations on inherited deformities of the feet in chickens says: "When the incubators are not running uniformly, many of the chickens are born with deformed feet. Such abnormality, however, is found with especial frequency in particular strains of birds, though eggs from other strains exposed to the same conditions may give perfectly normal results. The *liability* is the thing transmitted, but without the appropriate conditions the effect is not produced." That is to say, it is not the characters themselves that are inherited but the tendency to develop them. For example, we have the following example from plant genetics: Red and white primroses breed true;[67] but

66. Bateson: Lancet **2**: 453 (Aug. 16) 1913.
67. Siemens: Deutsch. med. Wchnschr. **45**: 1302, 1919.

if red primroses are raised in a greenhouse at from 30 to 35 C., the blossoms are all white and in appearance they differ not at all from white primroses. If after many generations of growing white flowers they are transplanted outdoors, they again breed red blossoms as before. Therefore, the red primrose cannot be said, when growing in hothouses, to transmit redness, but only the capacity to react to a certain environment by producing red pigment.

A striking illustration of what seems to be an example of another disease in mice that behaves exactly as if determined by a mendelian unit factor has been furnished by Hagedoorn-La Brand.[68] A breeding experiment was under way in which a colony of mice, composed of Japanese mice, large albinos, and hybrids of these two, was under observation. This colony was attacked by an epidemic staphylococcus infection, which killed all the Japanese mice but attacked none of the albinos, although the latter often ate the dead infected Japanese mice. No animals attacked by the infection recovered. *In the first hybrid generation, no mice died,* although quite as much exposed as the others, thus indicating that the resistance of the albinos depended on a single dominant genetic factor. This was further corroborated by the observation in the other generations of hybrid crossings. In the second hybrid (F 2) generation, of 125 animals ninety-one lived and thirty-four died, the theoretical expectation on the foregoing assumption being 93.75 to 31.25. Of crosses between F 1 and Japanese, of fifty-seven animals twenty-five lived and thirty-two died (theory, 28.5 to 28.5). Of crosses between F 1 and albinos, of fifty-one only one died (theory, no deaths). These results correspond remarkably well with the assumption that the albino mice possess one gene, the presence of which protects them against death from this infection, and which is lacking in the Japanese mice.

Presumably related to this sort of inheritable predisposition to an infectious disease is the inheritable deficiency in complement observed by Moore.[69] This deficiency is associated with a lack of resistance to infection, and it behaves as a simple mendelian reces-

68. Hagedoorn-La Brand: Am. Naturalist 54: 363, 1920.
69. Moore, H. D.: J. Immunol. 4: 1 s, 1919.

sive, hybrids of the first generation having as active complement as that of the normal parent.[70]

In Slye's experience, the hereditary factor of resistance to cancer may become so high in pure strains of selected mice that no ordinary amount of proliferative stimulus ever overcomes it, for strains of cancer-resisting mice have been developed which have never shown cancer during at least thirty generations, which would correspond in human life to close on a thousand years. Conversely, the capacity to resist cancer may be so effectively bred out of mice that virtually all of a selected strain develop malignant neoplasms from the ordinary proliferative stimuli occurring under even the best of living conditions. Only experiments, as yet not performed, will show whether the maximum experimental proliferative stimulation may overcome the resistance of pure noncancer strains of animals. Evidently in the heterozygous human race, maximum stimulation is almost always capable of overcoming such resistance as exists, since so large a proportion of people exposed to excessive amounts of roentgen rays have developed cutaneous cancer, but even so, we do not know of any particular form of tissue stimulation in man that leads to 100 per cent. cancer production. Conversely, cancer often develops in tissues in which there has apparently been no unusual amount of injury or stimulation; e. g., in man, cerebral gliomas occasionally develop after a definite trauma as an apparent sequel, but in most cases of cerebral glioma there is no history of trauma to the head, and in retinal glioma we see many cases occurring in a single family with no suspicion of any injury whatever to the retina. In this last case the hereditary influence is so great that merely the ordinary physiologic wear and tear seems to be sufficient to lead to malignancy.

C. *RELATION OF ANIMAL EXPERIMENTS TO HUMAN DISEASE*

As to the bearing of the experimental evidence on the problems of human cancer, the following facts must be considered:

1. Cancer in animals is, in all essential respects, the same disease as cancer in man.

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70. Hyde: J. Immunol. **8**: 267. 1923.

2. The laws of heredity are fundamental biologic laws applicable to every living thing, whether plant or animal. Mendel worked out the principles of inheritance with garden peas, and these principles have been found to hold good for all multicellular living things, whether plants or animals, whether peas or mice, insects or cows. This must inevitably be so, since all multicellular creatures take origin through fertilization of one cell by another, and since the fertilized cell produces quite the same sort of being as the one from which it came. If the laws of heredity established with peas hold good for mice, they should hold for men, for there is far less difference between mice and men than between mice and peas.

3. We have, furthermore, found that these mendelian principles do appear in human inheritance, although as yet we have no completely satisfactory evidence of mendelian inheritance in human cancer. Because man is a slow-breeding animal with very small families, it is not possible to study all sorts of inheritance in the human species, but there are some striking pathologic conditions which may be followed. For example, in hereditary hemophilia and in color blindness we find perfect illustrations of an inherited sex-linked recessive unit character. While we cannot usually secure sufficient data to test out the mechanism of human inheritance, Eugen Fischer [71] reports that in the crossing of Europeans with Hottentots, transmission of characters occurs in accordance with mendelian expectations.

Another illustration of human heredity conforming to the mendelian principles is seen in the separation into four groups of the human red corpuscles in respect to iso-agglutination.[72] Fischer, indeed, says that "all known normal and pathological characteristics that are transmitted by heredity follow the mendelian laws."

Therefore, if it is accepted that an important element in the occurrence of cancer in mice or other animals is the inherited character of the tissues, the same thing in all reasonable probability must be accepted for man. Furthermore, as pointed out previously, we do undoubtedly have instances in which the tendency to cancer has been inherited in man.

71. Fischer, Eugen: Naturwissensch. **10**: 650, 1922.
72. Ottenberg, Reuben: J. Immunol. **8**: 11 (Jan.) 1923.

Thus, among Warthin's carefully studied material appear families in which the cancer occurrence corresponds beautifully to the mendelian expectation, and in Levin's analysis of human cancer from a geneticist's point of view, the evidence led him to interpret it as indicating that resistance to cancer behaves as a dominant, susceptibility as a recessive character.

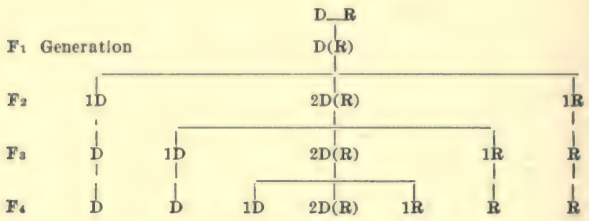

Fig. 2.—Typical mendelian inheritance: *D*, dominant; *R*, recessive; *D (R)*, presence of both factors, or heterozygous.

If we accept Maud Slye's contention that resistance to cancer behaves as a dominant character, and susceptibility as a recessive, we can understand the fact that human cancer commonly appears as an isolated condition in only a few members of a family, is often

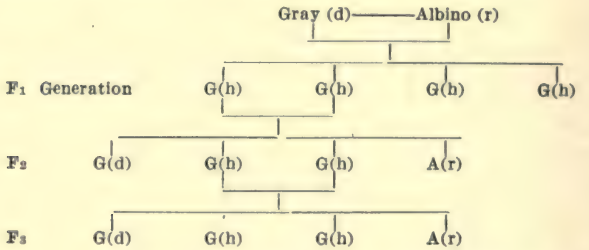

Fig. 3.—Typical mendelian inheritance in crossing gray and albino mice.

entirely absent in families of large numbers, and occasionally appears in a large proportion of the members of another family. As shown by the typical mendelian chart (Fig. 2), a recessive character does not appear in the first hybrid generation formed by crossing a recessive with a dominant character. If the heterozygous and dominant offspring of this generation are bred with dominant, the recessive never appears

(Fig. 4). When heterozygous individuals are bred together (Fig. 3), the recessive character does appear, but only in the ratio of one in four of the offspring. If two recessives are bred together, however, then the offspring all show the recessive character. If herterozygous offspring are bred with pure recessives, half the offspring exhibit the recessive character (Fig. 5).

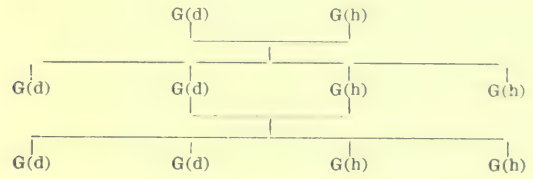

Fig. 4.—Inheritance in dominant mice bred with heterozygous mice.

Grayness is dominant, albinism is recessive. In the first hybrid generation the offspring will appear gray, but all still carry the recessive gene, i. e., they are heterozygous. When these are bred together, the mice of the F_2 generation yield offspring in the following proportions: one pure (dominant) gray which does not

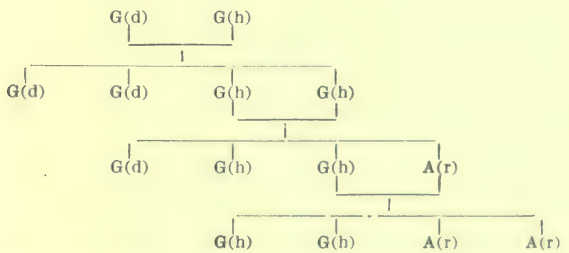

Fig. 5.—Way in which a recessive character reappears when heterozygous offspring are bred together, or when bred with recessives.

carry the gene for albinism, two heterozygous grays carying both genes, and one albino (pure recessive) which does not carry the gene for grayness.

In Figure 4, dominant mice are bred with heterozygous mice (derived from the crossing of gray and albino mice). As the dominant gray gene is always present in the offspring, they always appear gray, although half of them have the capacity to transmit

albinism when mated either with albinos or with heterozygous grays, as shown in Figure 5.

Transferred to the terms of human cancer, if this is a condition determined by a recessive unit character, then we should expect that in the heterozygous human race, breeding indiscriminately in respect to cancer ancestry, the recessive character of tumor susceptibility might crop out almost anywhere in a given family group, and ordinarily involve only a small proportion of the entire family. If, through chance, two individuals who inherited the tendency to cancer, which ordinarily would not manifest itself until after the reproductive period is over, should mate and bring forth offspring, it might be expected that these would all inherit the cancer susceptibility, and the demonstrated existence of "cancer families" agrees with this hypothesis. Equally well does the more common occurrence of families that show no cancer correspond to the expectations of this hypothesis.

The fact that either, but not both, of two parents had cancer, would not require that the offspring should develop cancer, since the other parent might be resistant (and dominant) to cancer (even if he or she had cancerous relatives), and the offspring might correspond to a first hybrid generation, none of whom have cancer. If one parent has cancer and the other parent is heterozygous to cancer, which should be the usual situation in the family history of descendants of a single cancerous ancestor, then the first generation should show some cancer cases (pure recessives) and some heterozygous offspring which do not have cancer themselves, but all of which may transmit the tendency to cancer. It is also perfectly possible for heterozygous and dominant resistant individuals to mate with one another for several generations without the recessive (cancer) appearing; but, when heterozygous offspring of such unions mate, cases of cancer might appear. This is illustrated by Slye in such a chart as the one here reproduced (Fig. 6). Here pure noncancerous (dominant), pure cancerous (recessive) and mixed (heterozygous) strains are shown extracted from common ancestors. Note how in the heterozygous strains (*C*), cancer crops out at intervals (16321, 20803) after three generations of direct ancestors with no cancer.

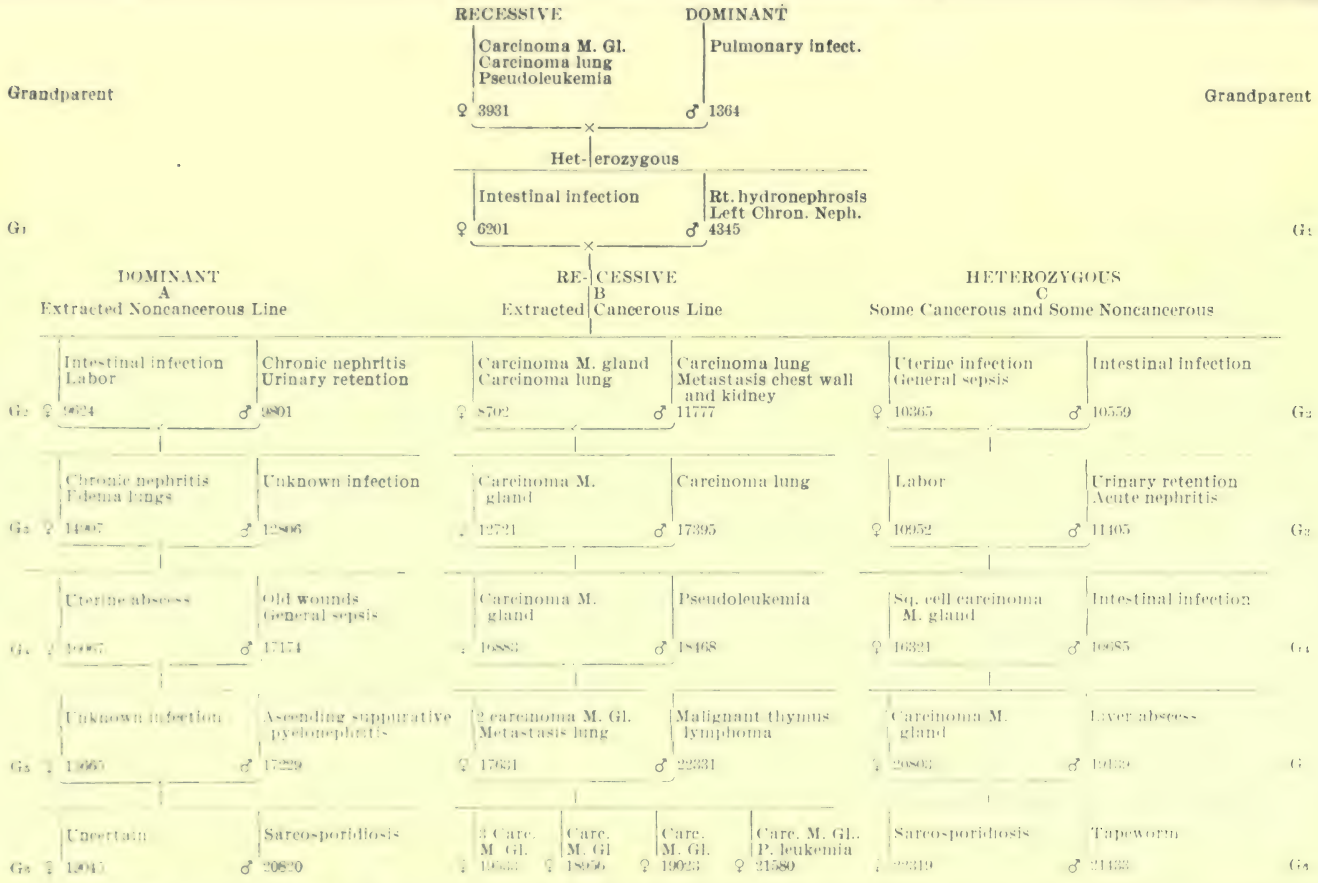

Fig. 6.—The segregating out of the noncancerous and the cancer tendencies, and their perfectly typical mendelian behavior in heredity. Strain 84, Br. II (Slye).

This heterozygous strain continued through (Fig. 7) exhibited in the thirteenth generation a mouse (30908) with lung tumor despite the fact that its direct ancestors had shown no tumors for eight generations. On the other hand, none of the mice of the dominant, cancer-resistant strain (*A*) ever exhibited cancer during fifteen generations for which they were followed, whereas the recessive, tumor-bearing strain (*B*) continued to produce mice succumbing to neoplasms in every generation.

With human families, cancer appearing at long intervals in such a strain as *C* would ordinarily be interpreted as an example of cancer appearing in an individual without cancer ancestry, which is entirely contrary to the fact. The heterozygous human being could transmit cancer tendencies unrevealed through an indefinite number of generations, provided it failed to mate with recessives or other heterozygotes; but when cancer did come out it would be as definitely inherited as if each ancestor for several generations back had had cancer. Failure to appreciate the manner in which inherited characters are transmitted has caused most of the confusion resulting from the impossible attempt to learn the hereditary relation of cancer by analyzing mass human statistics.

Certainly these considerations fit well with what we do know of human cancer. Until some one has carried out the arduous studies necessary to confirm or refute Slye's conclusions, as to the exact way in which the demonstrated influence of heredity is transmitted, it may be fair to consider them as at least offering a reasonable explanation of the influence of heredity on human cancer. It probably will be a long time before we shall have enough reliable information, controlled by necropsy records, to determine the influence of heredity on human cancer by direct observation. Perhaps the easiest way to test the validity of the hypothesis that in man, as in mice, resistance behaves as a dominant inheritable character and susceptibility to cancer as a recessive, will be by securing records of the offspring of matings in which both parents had cancer. Here we should expect that all, or, allowing for premature death, nearly all, of the offspring of such a union that reach advanced age would develop cancer. Such

statistics should not be impossible to secure, since they involve only two generations. But as yet, at least in America, we probably cannot find two generations in

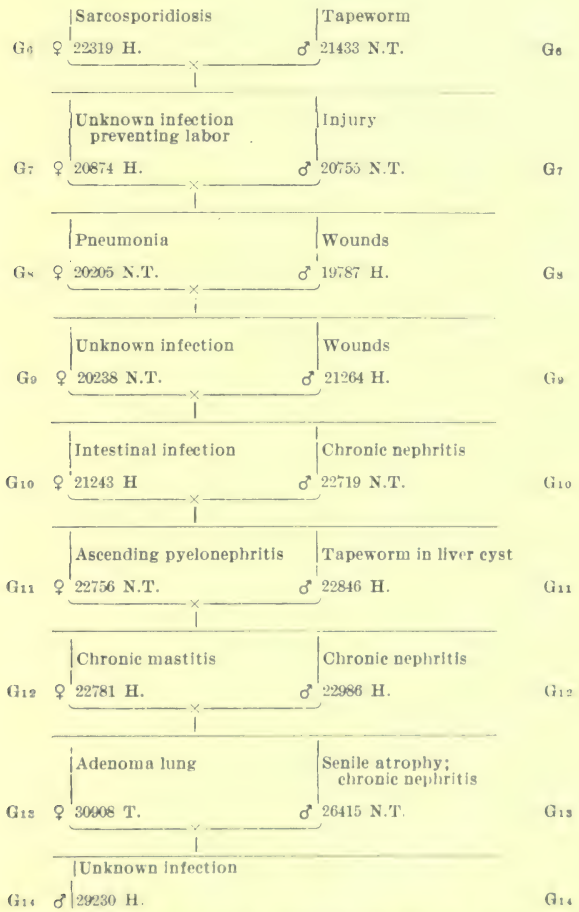

Fig. 7.—Heterozygous branch continued through the fourteenth generation; Strain 84, Br. C. (Slye): *H.* heterozygous; *T.* tumorous; *N.T.* nontumorous; *G.* generation.

which we can be sure of all the final diagnoses. Possibly in some more advanced country, such as Switzerland, where necropsies are more usual, the necessary data can be obtained.

SUMMARY

The coincident development of the sciences of genetics and experimental cancer research has begun to yield evidence bearing on the relation of hereditary influences to cancer occurrence. Human statistical evidence is not of sufficient accuracy or extent to render it of any value in the study of this subject. The occurrence of cancer families is unquestionable, but of doubtful value because of the possibility that such occurrences may depend solely on chance. Family occurrence of rare neoplasms, such as glioma of the retina, multiple neurofibromatosis, and multiple cartilagenous exostoses, cannot be dismissed as depending on chance. Human evidence being inadequate, we are compelled to rely on evidence from observations on animals. It is known that the principles of inheritance are the same in all species of animals as well as in plants, and that cancer, in its fundamental respects, is the same in man as in other mammals; therefore the drawing of conclusions in respect to heredity and human cancer from observations on experimental animals is justifiable. Such observations have shown repeatedly that an important element in the occurrence of spontaneous tumors in animals is determined by the heredity of the animals under study. Maud Slye has produced, solely through breeding, strains of mice that have never developed tumors in twenty and more generations, strains of mice in which the natural death of the adults is by cancer, and strains with less degrees of frequency of cancer occurring according to the mendelian expectation. In these animals the capacity to resist cancer behaves as a dominant character, the susceptibility to cancer as a recessive. There is evidence available which supports the inference that in man also the susceptibility to cancer behaves as an inherited recessive character.

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PRIMARY SPONTANEOUS TUMORS OF THE UTERUS IN MICE

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PRIMARY SPONTANEOUS TUMORS OF THE UTERUS IN MICE

WITH A REVIEW OF THE COMPARATIVE PATHOLOGY OF UTERINE NEOPLASMS. STUDIES ON THE INCIDENCE AND INHERITABILITY OF SPONTANEOUS TUMORS IN MICE. XIX

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One of the most striking differences between man and other mammals in respect to the comparative pathology of their neoplasms, lies in the great frequency of uterine tumors in the human female. Whereas cancer of the uterus represents one of the commonest of human neoplasms, in all other mammalian species it is a great rarity. The similar discrepancy in the frequency of cancer of the stomach we attempt to explain by differences in the diet or in the preparation of the food (heat, condiments), but the value of such an explanation is reduced when we consider the frequency of human uterine neoplasms. It is accepted that human uterine cancer is relatively more frequent in multiparae than in nulliparae, but most other mammals are much more multiparous than most women. Because of the bearing of this discrepancy on the general problem of cancer etiology, it has seemed worth while, before taking up the mouse uterine cancers specifically, to review the literature on the occurrence of uterine neoplasms in other animals.

Bovines: It is often stated that in this species more cases of uterine cancer have been observed than in any other domestic animal. Of 110 cases of malignant tumors in cows compiled by Sticker (1), seventeen or 15.5 per cent arose in the uterus, and this early paper established the view that in cows uterine tumors were by far the most frequent. Later observers have not had

the same experience, however. Thus, Trotter (2), in 305 cases of tumors in cattle found only one in the uterus, whereas 222 arose in the liver. Teutschlaender (3) compares these figures with a series of 1062 cases of cancer in the human female, of which 237 were in the uterus. Scholer (4) agrees with Trotter and others as to the infrequency of uterine cancer in cows. Since Sticker's compilation in 1902 of 17 cases, only one other recorded case could be found by Scholer, the case reported in 1912 by Wyssmann. Scholer himself has seen two cases of cylindrical-cell carcinoma of the bovine uterus, with metastases in each case. In an article in 1906, Trotter (5) also described a case of uterine carcinoma, and mentions having observed other cases. However, among 49 cancers in cows observed by Leo Loeb and Jobson (6), none was uterine. Also among 26 tumors in old cows examined by Murray (7) but one was in the uterus, an adeno-carcinoma.

On the other hand, Cadiot (8) in common with other French authors, considers the uterus a common site of cancer in cows, and certainly one cannot deny the statement that as far as uterine tumors in animals is concerned, the cow probably is most often affected. He says that among 77 malignant tumors in slaughtered cattle described by Detroye, five were uterine cancers. He quotes Bashford as having found at the abbatoir at Glasgow during 1902–3, five cases of cancer in old cows coming from Ireland.

Amond 417 cases of tumors of all sorts in bovines, Johne (9) found records of 7 leiomyomas of the uterus, certainly not a large proportion of this characteristic uterine growth; there were 6 other cases of leiomyoma in other organs. Wolff (10) adds to the above cases, one of a fibromyoma of the uterus weighing 55 pounds (Eckhardt), a case of sarcomatous infiltration of the uterus (Ostertag), a carcinoma of the cervix (Lucet), and two cases of uterine carcinoma with pulmonary metastasis (Barrier). Casper says that Kitt has found fibromyomas weighing 9 to 20 kilos, in the uterus of the cow, and in the older literature growths weighing as much as 100 kilos have been reported.

Since the above constitute all the uterine tumors that can be found recorded as occurring in cows, it is evident that in this species they are a great rarity, although of the total number of bovine tumors they constitute not an insignificant proportion. On the other hand, more uterine tumors have been observed in cows than in any other mammal except man, perhaps because with cattle an exceptionally large number of aged females are slaughtered.

We are indebted to Dr. L. E. Day for a statement as to the occurrence of uterine tumors in the vast material examined by him in the stockyards in Chicago, which bears out this statement. Among 204 tumors from cattle there were only six that arose in the uterus, of which two were fibromyomas, and one each diagnosed as adenoma, adenomyoma, carcinoma, and fibroma. This may be compared with 111 tumors from swine with no uterine tumors, and 47 tumors from sheep, one of which was in the uterus.

Horse. Here uterine tumors are indeed infrequent despite the advanced age these animals commonly reach. Sticker could find but 8 cases in his review of the literature until 1902, included in 509 tumors of all sites, the subcutaneous melanoma being the most frequent. To these but a few other examples may be added. Bland-Sutton (11) has seen two cases of sarcoma of the uterus, and Harrison (12) a very large fibromyoma. Roussy and Wolf (13) say that there are on record 18 cases of cancer of the uterus in horses, but give no further information. Eichler found no uterine tumors among 213 cases of tumors in horses, and Kimura (14) found only one carcinoma of the uterus among 142 equine tumors, of which no less than 49 were in the testicle.

Leiomyomas seem to be especially uncommon, for Johne (9) found none among his 250 equine tumors, of which 4 were leiomyomas of organs other than the uterus. Among 12 tumors in horses collected by Murray (7), none arose in the uterus.

Sheep. No record can be found in the literature of a uterine tumor in sheep, but among L. E. Day's 47 cases of tumors in sheep is included one case of leiomyoma.

Dogs. Although perhaps leading all the mammals in their susceptibility to neoplasms, dogs very seldom exhibit uterine tumors. Of 766 cases of tumors in dogs collected by Sticker (15) only four arose in the uterus. Among Murray's 48 tumors in dogs, were no uterine growths (7). Of Johne's 209 tumors in dogs, which included 4 myomas, only one was in the uterus. Williams (16) mentions only 3 cases of fibromyoma of the uterus in dogs, described by Keiffer, Wagner, and by Williams and Hobday. Wolff adds only one case, a "soft carcinoma" reported by Bruckmüller. Casper (17) found one cancer of the uterus among 51 cancers in dogs. Ichikawa (18) has described an old bitch with no less than 8 independent mammary gland tumors and 7 tumors of other organs, including 2 fibromyomas, one in the vagina and one in the tubal end of the uterus. Teutschlaender's tables include a myxosarcoma of the uterus of a dog, also a papilloma and a lymphosarcoma.

Roussy and Wolf (13) say that in dogs the uterus often contains myomas, less often cylindrical encephaloid epitheliomas, but they give no specific references.

Cats. Tumors of any sort are not common in cats, except the mixed tumors of the mammary gland, and the only uterine tumor of which we can find record is a fibroid described by Petit (19). Teutschlaender (3) includes in his table of recorded tumors a case of squamous-cell carcinoma and one of sarcoma of the uterus of 14 cats, but gives no details whereby the original reports can be located.

Swine. Here the only case that we can find reported is that of Leo Loeb (20), a carcinoma which weighed 16 pounds and produced metastases in the sublumbar lymph-nodes. Casper says that leiomyomas are not rare in pigs, Kitt having described one weighing 13 kilos. Of 92 benign tumors in swine compiled by Johne, 4 were in the uterus. Of 111 tumors from swine examined by L. E. Day, none was in the uterus.

Rabbits. In this species we find data of much interest in relation to the question of the influence of heredity in determining not only the occurrence of cancer, but its histological type and site of attack. Despite the large number of rabbits constantly

under observation in laboratories and subjected to post mortem examination, but few tumors have ever been found. Bell and Henrici (21) in 1916 could find but 35 tumors of all sorts on record as having been observed in rabbits, and added two cases of renal "nephroblastoma." To these may now be added: 1. An embryonal "nephroblastoma" reported by Scott (22). 2. Wallner (23) described a male rabbit, 5 years old, with generalized sarcomatosis of the abdominal viscera, origin impossible of determination, which was inoculated successfully in one rabbit by intravenous injection but could not be successfully re-inocuoculated. 3. A case of generalized lymphosarcoma reported by Aberatury and Dessy (24). 4. Marchand, in discussing the case reported by Schultze (25), mentioned a sarcoma, apparently arising in the mesenteric lymph-nodes, which was successfully inoculated into another rabbit but infection prevented further transplantation.

As with all other species of animals, the low incidence of tumors is at least partly explained by the fact that few old animals are examined. It is noteworthy that many of these tumors arose in rabbits over four years of age; the case of uterine carcinoma reported by Marie and Aubertin was in a 9-years-old rabbit. Despite this, however, it is certain that tumors must be infrequent in rabbits, in view of the number of animals under observation.

Of these 41 cases of tumor growths in rabbits no less than 24 arose in the uterus, a proportion excessively high because it includes the 13 cases reported by Stilling and Beitzke (26), which afford a valuable addition to the evidence previously presented from this laboratory, that hereditary influences are of importance in determining not only the incidence of cancer, but also the tissue in which the neoplastic tendency will express itself. This feature of Stilling's observations was reported after his death by Beitzke.

Stilling had been carrying on transplantation experiments, using a definite strain of rabbits which he raised himself. During these experiments he found tumors in the uterus of several animals. He waited years until he had accumulated thirteen

such animals and carried out a series of inoculation experiments with them. His death interrupted these experiments before he had given more than a few short communications concerning transplantable tumors before a local medical society. Beitzke examined the records he left behind him and reports them in this article. To quote this author: "He (Stilling) bought almost no rabbits, but raised his own stock, which not only permitted him to control the relation of his experimental animals, but in a certain sense to raise a whole tumor race. Unfortunately, no mention was found in his records that he had undertaken an exhaustive investigation concerning the influence of heredity." Stilling attributed his success to the fact that he kept his animals until they were old, the youngest tumor being in a four-year, the oldest in a seven-year rabbit. He also laid weight on the fact that most of the cases occurred in rabbits of the same strain. In the thirteen animals were found thirty tumors, twenty-five of which were adenomas or adenocarcinomas, mostly showing evidence of malignancy by infiltration, sometimes with areas resembling endothelioma. One of these tumors had many metastases in the lungs, spleen and peritoneum. Four seem to have been adenoma, and one a myoma. With two of the malignant tumors inoculation into other animals was unsuccessful, and with four animals in which autoinoculations were made no growths developed.

The other recorded cases of uterine tumors in rabbits are the following:

1. Wagner (27). The uterus contained five separate tumors, diagnosed as benign adenomas.

2. Marie and Aubertin (28). A single large "épithélioma cylindrique métatypique" without metastasis.

3. Katase (29). An adenocarcinoma of the uterus.

4. Selinow (30). A rabbit which had lived 6 years and 8 months after experimental removal of one kidney, exhibited in the uterus seven cauliflower-like growths, 2 to 3 cm. in diameter, which were considered to be carcinomas. The brief description available leaves doubt as to the true neoplastic nature of these growths.

5. Lack (31). The tumors found in the uterus and liver, following experimental dissemination of ovary cells, and believed by Lack to have resulted from this procedure, are considered by Shattock and others to have been derived from a primary carcinoma of the uterus.

6. Shattock (32). Reported the case of a rabbit with several nodular swellings in the mucosa of the uterus, so located as to suggest an origin in decidual tissues at the site of placentation. There were also nodules in the peritoneal cavity.

7. Leitch (33). Large multiple tumors, of adenomatous character, without any metastases. Inoculation of 19 rabbits did not produce any definite takes.

8–11. Boycott (34). In four rabbits among 140 to 150 killed for experimental purposes during one season, were found multiple adenomatous growths, apparently arising in decidual tissue left after abortions. These growths differ from those reported by Lack and Shattock, in the absence of metastasis. When metastasis is absent it is always difficult to tell whether such growths are true neoplasms or merely hyperplastic decidual remains.

In relation to Stillings' observations on hereditary influences, it is of significance that these four cases of Boycott's were all observed within a few months in a series of 140 to 150 female rabbits, although Boycott does not mention the possibility of relationship among these rabbits. Also, that the two cases of renal tumors reported by Bell and Henrici were, they say, "found on the same afternoon; and although we have autopsied over four hundred rabbits during the last three years, no other tumors have been seen." Although no record was kept to indicate whether these two rabbits were related, the coincidence of this occurrence makes relationship seem highly probable.

Rat. Despite the not infrequent occurrence of other tumors in this species, uterine growths are extremely rare. Teutschlaender's extensive compilation of animal tumors includes no cases; Wolff also mentions none, and the same is true of Williams. In their summary of the literature on tumors in rats, Bullock and Rohdenburg (35) include two cases of fibroadenoma of the uterus.

The extensive series of tumors in wild rats killed in plague work, and reported by McCoy (36) and by Woolley and Wherry (37), in all 125 tumors in about 123,000 rats, include but one uterine tumor, described by McCoy as an endothelioma, 2 centimeters in diameter, arising in the right division of the uterus.

The only other case of which we can find record was reported by Singer (38). This was a pea-sized nodule, resembling a leiomyoma but with large and varied cells, diagnosed as a spindle-cell sarcoma. It was inoculated into five rats, in one of which it grew, and subsequently it was carried for seven generations, with from 20 to 90 per cent of takes.

Other Wild Mammals. Numerous isolated observations are on record, of which the following seem adequately established:

Ground squirrels (*Citellus beecheyi*). Among 250,000 of these rodents examined in plague work, McCoy (39) found eight tumors. One of these was described as an alveolar sarcoma, measuring 1 x 2 x 4 centimeters, arising at the distal end of the right horn of the uterus. In another animal a tumor of similar structure arose either from the end of the right uterine division or from the right ovary.

Rhinocerus. Betke (40) described a remarkable case in a 40-years-old rhinocerus, in which the genital apparatus and the tumor tissue together weighed 120 pounds. There were nodules of myofibroma of the vagina, in the broad ligaments and under the serosa of the uterus. In the cervix was an extensive ulcerating adenocarcinoma which invaded the lymphatics of the uterine wall but produced no remote metastases. Also, Eber (41) is said to have observed multiple leiomyomas in the uterus of a rhinocerus.

Wild swine (*Sus scrofus*). An infiltrating scirrhous adenocarcinoma; no metastases mentioned (Fox) (42).

Armadillo. Six-banded armadillo (*Tetu novem cinctus*) with a fibroma uteri (Fox) (42). A similar growth in a nine-banded armadillo (Fox and Schumann) (43).

Elephant. Fibromyoma uteri, associated with calcareous fibromas in the ovaries of an Indian elephant (Fox and Schu-

mann) (43). Teutschlaender (44) states that he has seen a leiomyoma in the uterus of an elephant, and mentions one reported by Joest. No further details are given.

Black lemur (*Lemur macaco*). A very smal fibromyoma uteri (Fox and Schumann).

Canada porcupine (*Erethizon dorsatus*). Chorion epithelioma (Fox and Schumann).

Jaguar (*Felis onca*). This animal had not only a fibro-adenoma of the uterus, but a similar growth in the bile ducts and multiple cystic lymphangiomas in the omentum (Fox) (45).

Apes. Bland-Sutton (11) has described a myoma uteri in a baboon. A growth in the cervix of a monkey was described by Woods Hutchinson (46) as an adenoma or carcinoma.

Beaver. (*Myoptamus coypus*). Multiple myomas, described by Hilgendorf and A. Paulicki (47). There were three distinct uterine tumors and in addition "a large right-sided lymphatic struma and both adrenals were transformed into yellow tumors almost as large as the kidneys."

Birds. It may be proper to consider tumors of the oviduct in birds along with the uterine tumors of mammals, but despite the frequency of avian neoplasms these have seldom been described in the oviduct, although ovarian tumors are comparatively common. In their review of the literature, in which 160 cases of avian neoplasms are considered, Joest and Ernesti (48) mention in fowls: (1) a case of leiomyoma on the uterine ligament, which weighed 35 grams; (2) a round-cell sarcoma arising in the same site with metastases in the liver, spleen and kidney; (3) multiple adenomyomas of the wall of the oviduct; (4) a case, reported by Johne, of fibromyoma of the ligament; (5) a case of multiple round-cell sarcoma of the oviduct, described by Elsner; (6) a round-cell sarcoma which so involved the oviduct and the intestinal mucosa that the primary site could not be ascertained. They found no reports of carcinoma of the oviduct.

Curtis (49) has described, among 79 "tumors" found in fowls but apparently not studied microscopically, 10 in the oviduct wall but the neoplastic nature of these tumors is not established.

Fox (42) has reported a growth arising in an undulated grass-parrakeet (*Melopsittacus undulatus*) diagnosed as an adenocarcinoma of the oviduct; no metastases are mentioned.

In their work on the experimental production of tumors, Yamagiwa and Ohno (50) injected scarlet R oil into the oviduct of 41 fowls, and report that in three cases carcinomas resulted, with metastases in two cases.

Teutschlaender (51) mentions seeing a teratoma of the oviduct in a fowl, but gives no details.

Mice. Among the enormous numbers of mice examined in laboratories, surprisingly few tumors of the uterus have been found. In view of the remarkable functional activity of the uterus of this rodent such an immunity to neoplasms is indeed difficult to explain, especially when we consider the relative frequency of ovarian tumors in mice (52) and the enormous frequency of neoplasms in the mammary gland. Only the following cases can be found:

1. Fibromyoma uteri, reported by Haaland (53). The growth, "as large as a bean," was found in the left uterine horn of a 21-months-old mouse. In structure it resembled the human leiomyomas, but was somewhat more cellular and in one corner there were some glandular structures. This mouse also had a carcinoma of the breast.

2. Fibromyoma uteri, reported by Teutschlaender (54). He says that among 168 mice with 206 spontaneous tumors, all but four growths were epithelial. Among the four other tumors was a leiomyoma uteri in a mouse which also had a sarcoma of the vertebral column.

3. Carcinoma uteri, reported by Woglom (55). A mouse, 22 months old, of cancerous ancestry, with marked ascites, had a growth in the right horn of the uterus, with numerous metastases in the liver. Although the growth exhibited no glandular structure it was believed to be a carcinoma.

Uterine Tumors in the Slye Stock

Our own observations confirm the infrequency of uterine tumors in mice, at least in the strains under our observation,

for among 39,000 mice autopsied up to this time there have been found only the following undoubted primary uterine neoplasms:

11 cases of leiomyoma
7 cases of sarcoma
3 cases of adenoma
1 case of teratoma

22

Leiomyoma Uteri

These tumors correspond in all essential respects to those found in the human uterus (See Fig. 1), including the tendency

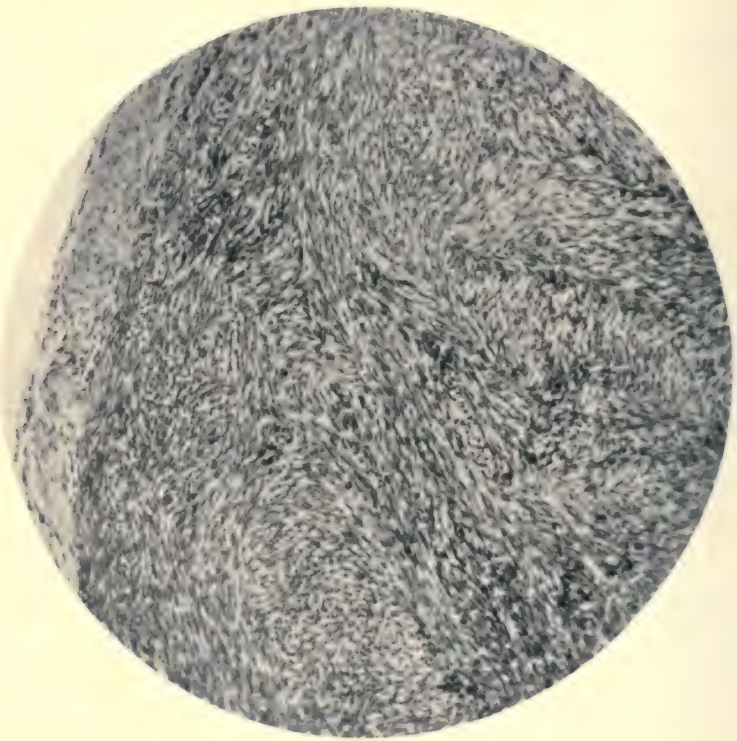

FIG. 1. TYPICAL LEIOMYOMA UTERI OF MOUSE (34931). × 125.

Showing the identity of the histological appearance with corresponding tumors of human tissues.

to be multiple, but they did not, in our specimens, reach as large a size proportionally as they often do in human females, our largest tumor being but 8 millimeters in diameter, and most of them were but 2–3 millimeters. Just how large a bean Haaland meant when he described his case, he does not indicate, but we suspect that it was larger than our 8 millimeter growth. Our specimens are mostly interstitial, and in no case have we any evidence that they had caused any harm. The proportion of muscular and fibrous elements varies, and these structures

FIG. 2. LEIOMYOMA UTERI UNDERGOING CENTRAL HYALINE DEGENERATION (18016). × 36.

The compressed uterine cavity is seen below the interstitial tumor, towards the center of which marked hyalinization is present.

show the same tendency to central hyalinization (See Fig. 2), necrosis, and calcification that is seen so often in human leiomyo-

mas (See Fig. 3). No evidence of malignant transformation was observed. In each of two cases there were two myomas, the other nine all being single tumor nodules.

FIG. 3. LEIOMYOMATA UTERI UNDERGOING CALCIFICATION (14570). × 25.

The compressed uterine cavity is seen below the tumor, with one greatly dilated tubule. The specimen has been decalcified, so the calcified areas are not deeply stained.

A striking feature was the coexistence of these uterine fibroids with other tumors, for among our eleven cases were six mice with mammary gland cancer, one with a papillary adenoma of the lung, and only four with no coexisting tumor. This is in interesting agreement with the fact that Teutschlaender's case of uterine fibroid was in a mouse with a sarcoma of the vertebral column, and Haaland's case was in a mouse with mammary gland carcinoma.

Sarcoma

As these tumors vary considerably from one another, they will be briefly described in detail.

10042. Bilateral spindle-cell sarcoma. This case was described in full in our paper on sarcoma in mice (56), with a photograph of the gross specimen. It was a bilateral growth, of large spindle cells, without metastases.

11828. Spindle-cell sarcoma, with metastasis in diaphragm (This was reported in the same paper). The tumor was composed of large spindle cells, with a metastatic growth in the diaphragm.

These two mice were closely related, 10042 being the half-aunt of 11828 through the male line.

12058. Sarcoma of uterus, with secondary growths in the left ovary and right kidney. The right horn of the uterus is replaced by a mass of soft tissue measuring 10 x 5 x 4 millimeters. In the left horn is a similar growth measuring 6 x 8 x 4 millimeters, and in the left ovary is a 10 millimeter mass, the right ovary being normal. At the upper pole of the right kidney is a mass of tumor tissue.

The portion of the uterus involved in the tumor growth is almost completely replaced, only isolated strands of muscle being found with tumor cells both inside and outside the muscular coat. No remains of the mucosa are present, and the lumen is filled completely by tumor growth, which also infiltrates the adjacent ligaments. There is no hemorrhage or necrosis. The tumor is composed everywhere of solid masses of moderately large polyhedral cells, with a round nucleus and considerable cytoplasm which is usually well stained. The cells occasionally form parallel rows, but show no marked tendency to assume any discernible structure. This growth resembles closely the tumors seen often in the testicle, adrenal, and ovary, and described by us as "mesotheliomas" in our papers on the tumors of these organs. Although in this case the ovary and kidney are involved, the gross features indicate that this tumor arose in the uterus.

15440. Sarcoma with metastasis in ovary and liver. A

mouse with two independent primary carcinomas of the mammary gland and an adenoma in the lung, had also a tumor of the right uterine horn, with extensive metastasis in the ovary and liver. Microscopically the uterine tumor tissue is found to be located chiefly within the mucosa, invading the muscular layer but little (See Fig. 4). The cells are round, with a dense nucleus

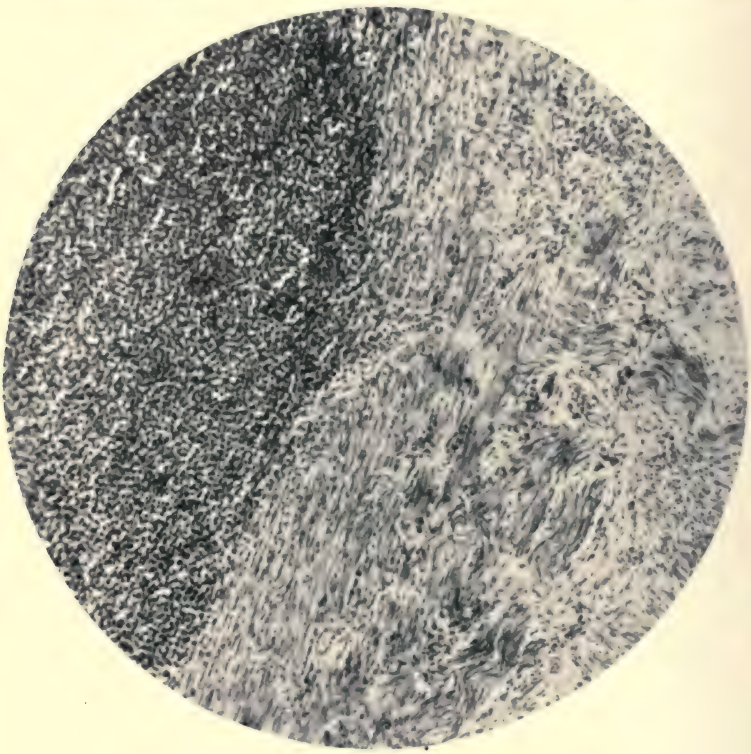

FIG. 4. SARCOMA OF UTERINE MUCOSA (15440). × 125.
The sarcoma tissue is seen infiltrating the muscularis.

and well defined cytoplasm, somewhat resembling the mesotheliomas. Similar tissue is found in the ovary and liver, and there is a slight invasion of the kidney by way of the perivascular lymphatic vessels.

17350. Spindle-cell sarcoma. The right uterine horn was greatly thickened, measuring 16 x 10 x 10 millimeters. It con-

tained a soft red tissue, but no exudate. There were no other tumors.

Microscopically the growth is composed of oval sarcoma cells, and seems to arise in the mucosa, infiltrating the muscular coats extensively and forming a dense layer under the serosa. The lining of the uterine cavity showed only tumor tissue with superficial necrosis.

22263. Mixed-cell sarcoma with extensive metastasis. This mouse had also four growths in the mammary gland, two being carcinomas and two apparently benign adenomas. There was a marked ascites, the liver was much enlarged and friable, a mass of large nodes occupied the mesentery. The wall of the uterus was greatly thickened by a tumor growth, and a secondary growth measuring 12 x 8 x 8 millimeters bound together the stomach, liver, pancreas and spleen.

The tumor tissue is composed of large oval or spindle cells with a moderate amount of cytoplasm, in some places resembling spindle-cell sarcoma, in others mesothelioma. The uterine wall is densely infiltrated with tumor cells, there being little muscle tissue left although most of the tumor tissue occupies the site of the mucosa, none of which remains. Invasion of lymphatic vessels by tumor cells is conspicuous in the uterine serosa and elsewhere. The ovaries, liver, and retroperitoneal tissues are invaded by masses of the same tumor tissue, and there is a slight invasion of the pelvis of the kidneys along the perivascular lymphatics. The spleen showed some amyloid infiltration.

37491. Round-cell sarcoma. A mouse which died with pneumonia exhibited a greatly thickened uterus, the lesion involving all parts of both horns, the walls of which were about 3 millimeters thick. There were no growths elsewhere. The uterus is diffusely infiltrated with a growth of large round cells with little cytoplasm, almost totally replacing the muscle tissue, although a few isolated uterine glands persist.

It will be noted that none of these sarcomas gave evidence of having been derived from a uterine myoma, and that metastasis was not usually extensive, in no case involving the lung. In

two cases (15440 and 22263) there were also carcinomas of the mammary gland.*

Adenoma

607. In a mouse which died of chronic interstitial nephritis with cardiac hypertrophy and hydrothorax, there was found in the left uterine horn a nodule measuring 10 millimeters in diameter. Microscopically this is found to be composed of a mass of tubular structures, lined with typical columnar epithelium, exhibiting varying degrees of papillary proliferation and cystic dilatation. The stroma of the growth is composed of large, deeply staining spindle cells, exactly resembling normal uterine mucous membrane connective tissue, without any evidence of collagenous fibril formation. The surface is everywhere covered with columnar epithelium which dips down into the stroma in glandular projections. Evidently this is a mucous membrane adenoma.

Infiltrating Adenomatosis

In two cases the entire uterine wall was diffusely infiltrated by a growth of typical tubules, which were abundantly distributed through the muscular coat and even under the serosa, but in which the character of the tubules resembled more a benign adenomatous growth than a carcinoma, despite the abundant infiltration of the uterine wall. Both of these mice had borne young, but not recently before death.

12477. In a mouse that died of chronic nephritis and had a cystic ovary, the uterus was diffusely enlarged, the horns being about 4 millimeters in diameter and bound down by adhesions so that it could not be detached from the adjacent tissue.

Microscopically the entire thickness of the muscular coat is infiltrated by tubules which resemble closely the normal mucosa tubules, even to the extent of usually showing about each tubule

* In one other case (28893) a mouse exhibited several small nodules in the right horn of the uterus. The lumen of the uterus contained purulent material, but post-mortem changes were too far advanced to permit of a positive diagnosis being made on the nodules in the wall. As far as can be determined they are composed of elongated cells somewhat resembling myomatous tissue, but are not so well encapsulated and are more cellular than typical myomas. Possibly they are sarcomatous, but the preservation is too poor to permit us to exclude granulomatous growth.

a zone of cellular connective tissue resembling that of the uterine mucosa (Fig. 5). The presence of these structures reduces

FIG. 5. DIFFUSE ADENOMATOSIS OF THE UTERUS (12477). × 60.

The glandular elements are seen to infiltrate the wall of the uterus to the serosa, with formation of some large cystic spaces. Despite the infiltrative character of the growth, the tubular epithelium retains a differentiated structure and usually a typical uterine submucosa structure underlies the tubules.

the amount of muscle tissue, which is distorted in its arrangement by the glandular islands. The tubules are irregular in shape, showing varying amounts of papillary proliferation and cystic dilatation, the latter being particularly marked beneath the serosa. There is no evidence of epithelial growth outside the basement membrane, and no inflammatory reaction. The adjacent tissues show no infiltration and the ovaries are normal. The kidneys show a chronic interstitial nephritis.

30784. A mouse which died of a subcutaneous abscess showed a diffuse thickening of both horns of the uterus, which was not adherent to any of the surrounding tissues. No other changes of importance were observed. The structure of this uterus duplicates exactly that of 12477, except that the amount of cellular connective tissue about the tubules is perhaps somewhat greater.

These two specimens resemble in some respects the uterine adenomyomas described by Cullen (57), especially in respect to the normal appearing glands and the typical submucous connective tissue about the tubules. They differ, however, in not showing any myomatous character and in the more diffuse infiltration, all parts of the uterus being invaded and many tubules being present beneath the serous surface, a condition not described in the usual human adenomyomas. So widespread is this glandular infiltration that one cannot be postitive that these growths are not malignant. The lack of extension outside the uterus and the preservation of the relation of epithelial structure to specialized submucous connective tissue, however, are strong evidences against malignancy.

Teratoma

10448. In the neck of the uterus was found a tumor 18 millimeters in diameter, partly hemorrhagic, partly white. The uterine horns were much distended with fluid. No other changes of importance were found.

Microscopically, the tumor consists of a mass of different sorts of tissue jumbled together without any discernible order or relation. Especially conspicuous are many islands of embryonic cartilage, besides which are noted atypical glandular and tubular structures, islands of squamous epithelium, small islands of atypical bone with marrow, areas of lymphoid tissue, imperfectly formed striated muscle fibers, masses of dense melanotic pigment, and areas suggesting neurolgia although no ganglion cells are recognized. There are no evidences either of infection or malignancy. This is evidently a very complex teratomatous tumor, derived from totipotential cells.

As far as we can learn, this is the third case of teratoma recorded in mice, there having been two very similar growths reported in our paper on ovarian tumors in mice (52), with illustration.

Carcinoma

It is indeed remarkable that among 39,000 mice, with at least 5000 primary spontaneous tumors, not one certain uterine carcinoma has been found. In addition to the two cases of diffuse adenomatous infiltration of the uterus described above, the malignant nature of which is not established, there is one other case in which we are not able to demonstrate positively whether a vaginal tumor arose in the cervix uteri or not, although the growth is not of a type comparable to those found in the human uterus.

38637. In a mouse with marked nephritis, hepatic abscesses, and a carcinoma of the mammary gland, there was found a hard rounded tumor 14 x 10 x 10 millimeters which completely filled the vagina nearly to the vulva and compressed the urinary bladder, which was pushed to the left, infiltrating also the tissues behind the cervix to the vertebral column, to which the growth was firmly adherent. The cervix was embedded in the upper part of the growth, but it could not be determined positively that the tumor arose in the cervix rather than in the vagina.

This tumor is a tubular carcinoma, somewhat resembling those found in the mammary gland, but having a better developed stroma than such tumors usually have; it is quite unlike any ordinary tumor found in the human uterus and is altogether different from the mammary gland tumor of the same mouse, which is composed solely of closely packed tubules with virtually no stroma. The vaginal tumor shows some tendency to cystic dilatation of the tubules, numerous areas of necrosis, and a free infiltration of the adjacent tissues. The diagnosis is glandular carcinoma, but whether of the cervix or the vagina we are unable to state.

Secondary Tumors

These are very rare, there being practically no intra-abdominal carcinomas in mice to produce implantation metastases. None of the many cases of mammary gland carcinoma has led to uterine metastasis. In a few of the cases of retroperitoneal tumors there has been more or less infiltration of the uterus by direct extension. In mouse 12744 a tumor of mesothelioma type arising in the adrenal produced numerous intraperitoneal growths, which were found attached to the uterus and liver. In 19061 a tumor of similar structure, but so widely diffused that its point of origin could not be determined, invaded the uterus as well as the ovaries, kidneys, pancreas, and liver. In 21152 a retroperitoneal lymphosarcoma invaded the perirenal tissues, kidneys, urinary bladder, and uterus. In some of the cases of leukemia and pseudoleukemia the uterus is found extensively infiltrated, as are the ovaries and other tissues.

SUMMARY

A review of the literature on the comparative pathology of uterine tumors emphasizes the great infrequency of such growths in all the lower mammals, as contrasted with the high incidence in man, and even in proportion to the occurrence of tumors in other sites in the lower mammals. This is particularly noteworthy in view of the great frequency of mammary gland carcinoma in some species, and of ovarian tumors in other animals. Of all lower mammals uterine cancer is least rare in cows, but even in this species it is far from common and constitutes but a small proportion of all the bovine neoplasms. Even in dogs, susceptible as they are to tumor formation, uterine carcinomas are almost unknown. A few cases have been described in wild animals.* Leiomyomas are observed perhaps a little more frequently than carcinomas, but they are remarkably uncommon compared with their great frequency in the human female. Only in rabbits are uterine tumors at all numerous, and this

* While this article was in press, " Disease in Captive Wild Animals " by Herbert Fox (Lippincott, 1923) has appeared. Here it is stated that among tumors observed in the Philadelphia Zoological Garden were eight involving the uterus, but these are not described in detail. Most of them are included among the cases reported elsewhere by Fox and referred to previously in this article.

because one observer (Stilling) had thirteen cases in a single strain of rabbits under his observation, a fact supporting the experience of this laboratory that heredity may determine not only the frequency but also the site and character of tumor formation.

In mice but few uterine tumors have been described. In the literature we can find records of but three; two fibromyomas and one carcinoma. They are also very rare in the Slye stock, for in 39,000 autopsies we have found but twenty-two animals with unquestionable primary uterine tumors, of which eleven were leiomyomas, seven sarcomas, three adenomas, and one teratoma. The leiomyomas were all rather small, and exhibited quite the same character as in the human uterus, with the same tendency to hyalinization and calcification; in none did we observe any malignant transformation. The sarcomas were of different types, some spindle-cell and others with comparatively large polyhedral cells, these growths resembling somewhat the "mesotheliomas" of the urogenital tissues. Two of the adenomas exhibited a diffuse infiltration of the entire uterine wall, suggesting malignancy, but the histological characters resembled those of the human adenomyomas. There was also a carcinoma filling the vagina, which may have arisen in the cervix. With these possible exceptions there were no cases of carcinoma of the uterus, either primary or secondary, in all these 39,000 mice, despite the great number of other neoplasms among them. Neither was anything found resembling a syncytioma, either in the uterus or elsewhere.

REFERENCES

(1) Sticker: Arch. f. klin. Chir., 1902, lxv, 616.
(2) Trotter: J. Comp. Path., and Therap., 1911, xxiv, 1.
(3) Teutschlaender: Ztschr. f. Krebsforsch., 1920, xvii, 343.
(4) Scholer: Ztschr. f. Krebsforsch., 1915, xv, 193.
(5) Trotter: J. Comp. Path. and Therap., 1906, xix, 11.
(6) Loeb and Jobson: J. Comp. Med. and Vet. Arch., 1909, xvi, 385.
(7) Murray: Third Sci. Report, Imperial Cancer Research Fund, 1908, p. 11.
(8) Cadiot: Bull. de l'Acad. de med., Paris, 1921, S. 3, lxxxv, 396.
(9) Johne: Quoted by Casper, Lubarsch-Ostertag, Ergebn. d. allg. Path., 1907, II², 1102.
(10) Wolff: Die Lehre v. d. Krebskrankheit, 1913, iii, 242.

(11) Bland-Sutton, J. B.: J. Anat. & Physiol., 1885, xix, 415.
(12) Harrison: Cited by Wolff, l. c. p. 230.
(13) Roussy and Wolf: Ann. de méd., 1920, viii, 464.
(14) Kimura: Gann, 1917, xi, 38.
(15) Sticker: Arch. f. klin. Chir., 1902, lxv, 689.
(16) Williams: Natural History of Cancer, New York, 1908, p. 96.
(17) Casper: Arch. f. Tierheilk., 1893, xix, 14.
(18) Ichikawa: Gann, 1917, xi, 1.
(19) Petit: Quoted by Casper, l. c.
(20) Loeb: Tr. Chicago Path. Soc., 1900, iv, 287.
(21) Bell and Henrici: J. Cancer Res., 1916, i, 157.
(22) Scott: J. Cancer Res., 1917, ii, 367.
(23) Wallner: Ztschr. f. Krebsforsch., 1921–2, xviii, 215.
(24) Aberatury and Dessy: Cited by Wallner (23), without reference or further details.
(25) Schultze: Verhandl. d. deutsch. path. Gesellsch., 1913, xvi, 358.
(26) Stilling and Beitzke: Virchows Arch., 1913, ccxiv, 358.
(27) Wagner: Centralbl. f. allg. Path., 1905, xvi, 131.
(28) Marie and Aubertin: Bull. Ass. Franç. pour l'étude du Cancer, 1911, iv, 253.
(29) Katase: Verhandl. japan. path. Gesellsch., ii, 89.
(30) Selinow: Abst. in Centralbl. f. Allg. path., 1908, xix, 122.
(31) Lack: J. Path. & Bacteriol., 1900, vi, 154.
(32) Shattock: Tr. Path. Soc. London, 1900, li, 56.
(33) Leitch: Proc. Royal Soc. Med., (Path. Sect.) 1911–12, v, pt. 3, p. 1.
(34) Boycott: Proc. Royal Soc. Med., (Path. Sect.) 1910–11, iv, pt. 3, p. 225.
(35) Bullock and Rohdenburg: J. Cancer Res., 1917, ii, 39.
(36) McCoy: J. Med. Res., 1909, n. s. 16, 285.
(37) Woolley and Wherry: J. Med. Res., 1911–12, n. s. 20, 205.
(38) Singer: J. Path. & Bacteriol., 1913–14, xviii, 495.
(39) McCoy: J. Infect. Dis., 1914, xiv, 53.
(40) Betke: Frankfurter Ztschr. f. Path., 1911, vi, 19.
(41) Eber: Deutsch. Ztschr. Tiermed., xviii, 316. Cited by Wolff, III[1], p. 271.
(42) Fox: J. Path. & Bacteriol., 1912, xvii, 217.
(43) Fox and Schumann: J. Comp. Path. and Bacteriol., 1915. xxviii, 298.
(44) Teutschlaender: Ztschr. f. Krebsforsch., 1920, xvii, 327.
(45) Fox: Zool. Soc. of Philadelphia, 49th Ann. Report, 1921, p. 36.
(46) Woods Hutchinson: Cited by Williams, loc. cit., p. 93.
(47) Hilgendorf and Paulicki: Virchows Arch., 1870, xlix, 295.
(48) Joest and Ernesti: Ztschr. f. Krebsforsch., 1915, xv, 1.
(49) Curtis: J. Agric. Res., 1915, v, 397.
(50) Yamagiwa and Ohno: Gann, 1918, xii, 3.
(51) Teutschlaender: loc. cit., p. 323.
(52) Slye, Holmes and Wells: J. Cancer Res., 1920, v, 205.
(53) Haaland: Fourth Sci. Report, Imperial Cancer Research Fund, 1911, p. 11.
(54) Teutschlaender: loc. cit., p. 325.
(55) Woglom: Proc. New York Path. Soc., 1919, xix, 60.
(56) Slye, Holmes and Wells: J. Cancer Res., 1917, ii, 1.
(57) Cullen: Bull. Johns Hopkins Hosp., 1897, vi, 133.

THE CLINICAL USE OF INSULIN.

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Between June, 1922, and April, 1923, insulin was employed interruptedly or continuously in the course of treatment of some 150 diabetic patients. This report presents in brief the results obtained and comments that seem to have been warranted.

Material: The preparation of insulin was begun in this laboratory in June under the direct management of Dr. E. J. Witzemann. The method was essentially that developed by Collip and placed at our disposal through the courtesy of the Toronto group. A portion of the insulin used later was supplied by the Eli Lilly Company, and later still all of the material came from that source.

Route of Administration: Experiments were conducted with oral, rectal, vaginal, intranasal, intravenous, and subcutaneous administrations. Inunctions were also tried. Many variations were attempted in connection with each. Positive effects were obtained with subcutaneous and intravenous injections, very weak, doubtful or frankly negative results with the others. For practical clinical purposes subcutaneous injections were used to the exclusion of other modes of administration.

Technique of Hypodermic Injection and the Number and Time of Doses: Multiple doses have been largely eliminated and as a routine the single morning injection has been adopted for over 90 per cent. of all cases. It still happens occasionally that a case will be given 2 doses per day, one before breakfast and a smaller dose before the evening meal. Patients admitted in emergencies have been given insulin in divided doses to meet rapidly changing initial conditions. Finally all such cases have been reduced to one or two doses. The morning dose is given 30 minutes to 1 hour before breakfast. A few receive the dose 1 hour and 15 minutes before breakfast. These are cases which tend to show glycosuria after breakfast and an insulin reaction before the noon meal. With these it has seemed desirable to permit time for the insulin to absorb and thus secure a better registration of the insulin

action curve with the curve of glucose supply that follows the meal.

Preference has been given to insulin preparations containing at least 20 rabbit units per c.c. of volume. More concentrated preparations (e.g. 30 units per c.c.) have been used in some cases to reduce the volume of the injection and prolong the action curve. At present 20 units per c.c. is the strength employed by preference in the routine practice.

We have preferred 27 to 29 gauge hypodermic needles ¾ to 1 inch in length with 1½ to 2 c.c. glass syringes. The needles are inserted full length *under* the skin into a loose space. Pressure during injection is fastidiously avoided. The solution is warmed before injection. A flexor or adductor surface where the skin is thin and elastic and the subcutaneous space commodious is preferred. Care is taken to avoid raising a lump or welt at any time during injection. The needle point should be freely movable under the skin and the skin freely movable over the point of the needle. If it lies *in* the skin or in the firmer tissues beneath, pressures may arise during injection with unnecessary pain, soreness, scarring, necrosis, etc. Patients are taught to give their own injections whenever feasible. The thigh is then usually selected. If resistance is met the needle is readjusted until the fluid flows in with no more than the gentlest pressure of a finger tip on the plunger.

Method of Beginning and Advancing the Insulin Dosage: Various methods have been employed in the effort to find the most direct and expeditious. A number of preliminary plans were discarded after trial. Different cases present different problems and no one stereotyped procedure has proved equally effective in all. When a new case presents for management, the history and the first inspection and examination of the case permit one to judge of the type and degree of the diabetes and these determine the initial procedure. For illustration, assume that the patient is a young man of 30, that he looks emaciated and has the peculiar translucent pallor suggestive of a severe diabetes. He states that he has had the disease to his knowledge for only one year, that a year ago he weighed 160 lb. Now he weighs 110 lb. The disease has disabled him in a year. Unless the emaciation is attributable to enforced undernutrition such a case is obviously one of inherently severe diabetes. By diet adjustment alone nothing short of rigid restriction promises to restore him

to the non-diabetic status. In such a case there would seem to be no object in temporizing with intermediate diets. He is placed at once on a simple measured ration containing 0.5 to 0.8 gm. protein and 2.0 to 2.5 gm. fat per kg. of body weight plus the least amount of carbohydrate that can be given without making the diet unpalatable; for example:

	gm.	C. gm.	P. gm.	F. gm.	
Cream, 20 per cent.....	400	20	12	80	
Eggs	2		12	12	
Bacon	30		5	15	
Butter	20			17	
Total		20	29	124	Cal. 1312

At bed rest this suffices for caloric maintenance of the 50 kg. patient and commonly for a nitrogen balance. The glucose value of the diet estimated as C + .58 P + .1 F is 49 gm. On this diet either the urine becomes sugar-free or it does not. If it becomes sugar-free the diet is increased sufficiently to give it a glucose value some 25 im. higher, i.e. to 74 gm. or thereabouts. If this produces no gross glycosuria the diet is advanced again, and so on. Either on the initial diet or a later one there occurs a steady excretion of 15 to 25 gm. of glucose per day and one learns by the difference between the glucose supply and the glucose excretion the number of grams of glucose utilized per day, i.e. the existing tolerance "T" or the power to burn glucose. Having established a steady excretion of, say 10 to 25 grams of glucose per day, the probable dose of insulin necessary to check it can then be calculated. It has been our experience that 1 unit will account, directly, for 1.0 to 1.5 gm. of glucose. If this individual excretes steadily 25 gm. of glucose daily for 3 days, 20 units of insulin will approximately suffice to make the urine sugar "free." He then receives ½ of this calculated dose. First 1 drop of the preparation is injected intracutaneously as a sensitization test, and thirty minutes later the remainder of the 10 units (ordinarily ½cc.) subcutaneously. This is done as a rule in the afternoon and the 24 hours' urine closing the next morning usually shows a diminution of the glucosuria. In any event, barring the possibility that the initial 10 units causes an insulin or a protein reaction (rare) the patient receives 10 units of insulin before breakfast on the day after the initial test. The urine for that

day indicates the effect. On the following day or the next, if the first day's results are not unexpectedly favorable, the morning dose is increased by 5 or 10 units, making the total injection 15 to 20 units, or if desired one may wait for a day or two for the figures to become steady before raising the dosage. Whichever plan is followed, the glycosuria ultimately falls to 2 to 5 gm. per day or thereabouts. At this stage, without waiting for complete desugarization, one may increase the diet again by a measured number of grams G, again establish a steady excretion, and again raise the insulin dosage by an amount equal to ½ of that calculated as necessary for complete desugarization; or, one may advance the diet and the insulin coincidently to save time. We first proceeded in the first way; later, knowing better the value of the preparations, more often in the latter way. There is an advantage in the latter in that the establishment of a rather heavy glycosuria tends to retard or beat down a rising natural tolerance, thus necessitating more time and insulin to accomplish what is desired than is the case if the patient is never permitted to develop a heavy glycosuria. But the former method would seem to be safer and to give more exact information, so that for one beginning the use of insulin it is preferable. In such a case as that under discussion the patient's own tolerance may begin to rise under the effects of relief from the excess of sugar in circulation, and it may rise so rapidly that what is for the moment a necessary dose of insulin becomes an overdose. A further increase in such a situation is naturally undesirable.

As the diet is increased stepwise the time comes when a decision must be made as to the limit. This will depend upon circumstances. Injections of a volume of more than 3 cc. of any strength daily offer mechanical difficulties and the mere volume necessary may determine a practical limit. It is preferable to keep the volume within 1 or 2 cc. per day for the long run. When using 20 unit material this is a dose of 20 to 40 units. The cost is also an item. It has been our practice to consider the size of the patient and the nature of his life and work. If a diet containing the equivalent of 120 gm. glucose and 18 times that number of calories (i.e. 2100 calories in round numbers) offers a physical and economic solution of that patient's problem he is placed on such a diet if possible with the least loss of time. A larger man doing more physical work may be unable to follow his occupation on a diet with a G of only 120 and may require

140 or more if he is not to become financially dependent. This man should receive a satisfactory diet to solve his own particular problem. Again, if a man requires 140 and can burn 120 without insulin, he needs very little insulin anyway to bridge the gap between full working power and disability, but if he is able to burn only 50 gm. without insulin he will require insulin equivalent to 90 gm. to solve his problem. This would entail the daily use of perhaps 4 to 5 cc. of the product (at 20 units per cc.) and the volume itself may deter one from proceeding so far.

Cases in which by diet adjustment alone it is possible to preserve life, health and working power without disability have not been considered as insulin cases.

Temporary Uses of Insulin: Many cases present in which diet adjustment offers a solution but for which time can be saved by the temporary use of insulin. Thus, to desugarize and restore tolerance in a certain case may require 2 to 3 weeks on diets below that on which the case leaves the hospital. The undernutrition and the time and money spent on the partially disabling regimen can be avoided by the interrupted use of insulin. Desugarizing the patient by means of insulin on a higher diet permits in suitable cases a restoration of natural tolerance, like that attainable through desugarization by diet depression but with less discomfort to the patient. We have frequently given insulin to hasten desugarization or in lieu of cutting the diet below a certain level. After desugarization the insulin has then been withdrawn gradually or abruptly with no return of glycosuria.

Insulin in Pre-coma: In cases of severe acid intoxication the practice has been to institute a diet containing protein 0.5 to 0.75 gm. per kg., fat 2.0 to 2.5 gm. per kg., with the carbohydrate as low as feasible. The patient is given at once 20 gm. of the bicarbonate of soda by mouth, after which a reading is made of the volume percentage of CO_2 liberated from the plasma when examined by the Van Slyke method (i.e. the plasma bicarbonate or CO_2 combining power). Soda is then given by mouth at the rate of 15 to 20 gm. per hour until the blood plasma shows a reading of 40 to 45 vol. per cent. CO_2 by Van Slyke, or until from the symptoms and signs this procedure is deemed unnecessary. When insulin is used effectively one usually stops the soda after giving only 20 to 60 grams. In certain severe emergency cases in which it is impossible to know in advance what the glucose excretion will

be in the first 6 to 12 or 24 hours after admission, insulin has been given to check acid production without knowing how much sugar the patient will excrete in a given period of time. In such cases large doses (40 to 80 units) have been given quickly with the view to providing for the oxidation of 40 to 80 gm. of glucose in excess of any that might burn without insulin, in order to annul acid production. To insure against over-dosages of insulin under such conditions sugar has been given by stomach prior to the administration of the insulin, and thereafter in quantity sufficient to provide all of the insulin given with enough glucose to act upon without making any allowance for the glucose that might arise from the diet and endogenous sources. Thus, if a single dose of 60 units of insulin were calculated to throw into oxidation 60 to 90 gm. of glucose in 10 hours, or on the average 6.0 to 9.0 gm. per hour, then at least that quantity of sugar has been given hourly to insure against the possibility that the glycosuria was on the decline when the insulin was given and would fade out rapidly thereafter, leaving the patient with not enough glucose to counteract the insulin given. This procedure has been used only as a temporary emergency measure. It may be superfluous to mention that diabetic patients in acid poisoning who have received no insulin not infrequently before death pass urine free of sugar and acetone bodies (provided no infection complicates the case) simply as the result of the limitation of the intake of food occasioned by the general condition. To give insulin to a patient naturally approaching this juncture and without giving sugar might lead to serious insulin poisoning. With cases in which as a result of infectious complications the overproduction of acid tends to continue in spite of diet restrictions, insulin has made it possible to stop acid production and thus avoid the continued use of alkali, besides permitting of adequate nutrition. This has proved of very great value and especially in complicated cases.

Diagnostic Use of Insulin: Certain cases of the general order of those that are called renal glycosuria have been treated with insulin. A certain case shows steadily 2 to 3 gm. of glucose in the urine on a diet with a glucose value of 100 gm. On a diet with a glucose value of 200 gm. he passes on the average 4 to 6 gm. of sugar in the urine. With the dietary G equal to 400 he passes 8 to 10 gm. He has few of the ordinary symptoms of diabetes, the sugar having been discovered during an insurance examination. The blood sugar percentage ranges between 0.1 and 0.12.

Doses of 5 and 10 units of insulin have exerted little effect on the glycosuria in several such cases. This observation with variations, made on a number of cases showing no sharply definable tolerance limit, suggests the possibility of using insulin in differential diagnosis.

Determining the Value of a Given Preparation of Insulin: Individuals who have long suffered from severe diabetes and who for a period of years have not under any circumstances developed more than a certain maximum tolerance afford the most favorable medium for accurately gauging the number of grams of glucose that will be thrown into utilization by a given volume of insulin solution. Individuals whose glucose tolerance is known to be subject to fluctuation in response to factors other than insulin administration are not suitable cases to employ for the purpose of standardizing preparations. Again, early or mild cases of any kind are not dependable because if they have fixed or relatively fixed tolerance limits there is no way of demonstrating this except by the passage of time. Complete or nearly complete desugarization of the urine in any case of diabetes may be and commonly is followed by a lesser or greater increase of natural tolerance whether the desugarization be effected by diet adjustment alone or diet adjustment plus insulin. Hence if a certain diabetic individual on a fixed diet be excreting steadily 10, 20 or 30 gm. of sugar in the urine daily, and if after receiving a certain volume of insulin solution he pass urine that is sugar "free" or nearly so, it does not follow that the insulin injected has accounted directly and *per se* for all of the glucose that has disappeared from the day's urine. The desugarization or radical reduction of circulating sugar may result in a rising production of insulin from endogenous sources, and this endogenous insulin by adding its effect to that of the insulin actually injected from without may make it appear that the latter has a greater potency than it actually has. With these considerations in view it has seemed appropriate to select with great care for standardizing purposes old cases with long metabolic records, whose tolerances have long remained fixed or relatively fixed at low levels. Such cases have then been placed on regimens which permit them to excrete steadily 20 to 30 gm. of sugar per day. They have then been given doses of insulin that reduce the sugar excretion definitely but without approaching complete desugarization. If 10 units of a preparation given under such conditions lowers the

glucose excretion from an average of 25 to an average of 15 gm. per day it may be assumed that 1cc. has accounted for the utilization of 10 gm. of glucose. If the dose of insulin is then discontinued while other conditions remain as before, and the sugar excretion then rises to its former level, confirmatory evidence is added. However, should the glucose excretion remain depressed after withdrawing the insulin, this would suggest an improvement of tolerance and the test would be indecisive. Working with selected cases and in this way, we have found that 1 rabbit unit increases glucose combustion by approximately 1.0 to 1.5 gm. with considerable consistency.

Cases have been seen in which 1 unit reduced glycosuria by as much as 3.0 gm. per unit and others, especially mild arteriosclerotic cases and cases of so-called renal glycosuria, in which insulin exerted remarkably little effect.

Results: Since beginning the use of insulin one case has died of pneumonia although free of diabetic symptoms during the disease. One case with an empyema died during management suddenly before the glycosuria was eliminated and while hyperglycemia still prevailed, in cardiac collapse. One case received in extreme inanition and unable to speak because of weakness, but free of glycosuria, was given a large increase of diet and a calculated dose of insulin. On admission the blood sugar reading was not made. This case developed hypoglycemic convulsions and the blood sugar was then at a level too low to read. Sugar administrations restored a hyperglycemia and gross glycosuria, but the patient died without regaining consciousness. Subsequently a similar diabetic case received on another service died a similar death with a similar absence of blood sugar but without having received insulin. Williams of Rochester has described an analogous case of fatal hypoglycemia with convulsions due to inanition without insulin. Our own case was diagnosed as one of fatal hypoglycemia induced by an over-dosage of insulin. In retrospect, it would seem probable that the patient had been gravely hypoglycemic before the insulin was given, and had the diet been increased until glycosuria had recurred before giving the insulin the fatality probably would have been averted. The precarious state of the patient made it seem necessary at the time to hurry with extra food and the means to utilize it. The case is cited to illustrate the danger of even a moderate overdose of insulin in a profoundly undernourished case. Except for the

above mentioned case, all of the patients so far treated are living, on diets that fulfill their legitimate needs for food.

Shall the Insulin Case be Kept Sugar-free?

Old cases with low fixed tolerance limits have been found difficult to keep sugar-free at all times, owing to unavoidable variations of diet, insulin dosages, exercise, etc., and it would not seem necessary that they do so, since they seem to have no natural tolerance to lose. On the other hand, all cases that have a considerable natural tolerance or power to regain such should be kept sugar-free if possible, and it has been found to be possible in most cases.

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SOME MILDER FORMS OF DIABETES WITH SPECIAL REFERENCE TO MILD DIABETES IN ELDERLY PERSONS WITH ARTERIOSCLEROSIS*

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In these days of insulin and unprecedented interest in diabetes, when much has been written and said about coma and the severer cases, it may not seem amiss to speak for a few moments on another phase of the diabetic problem, one of special importance to the practicing physician, but also, I think, not devoid of theoretical interest. I refer to some of the milder forms of diabetes and in particular to the management of elderly individuals with arterial disease and a mild diabetes.

Elderly people with arteriosclerosis, like people of all other ages, may of course show different types of diabetes. The fact that a patient is elderly and that he has advanced changes in the blood vessels does not by itself fix the type of diabetes that he may have. But in elderly people with arterial disease there is a type of diabetes which recurs with great fre-

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quency, and it is to this type that I refer. The patients may be men or women, but are more often men. They are usually in the fifties or sixties when recognized as belonging to this group, but they may give a history of glycosuria extending back to the forty-fifth year, even to the fortieth year of life, possibly in an exceptional case even into the late thirties. If the onset is in the early forties and if the case is seen at that time it may not be possible to recognize it as one destined to run a slow, mild course. However, when seen later in the light of past events it may be perfectly apparent that the case was characterized from the beginning by inherent mildness and by a tendency to progress in severity very slowly or not at all in spite of laxnesses of treatment or flagrant abuses that would inevitably make anything but an inherently mild case permanently more severe. Patients of the type to which we refer when they present themselves for treatment may have no symptoms of diabetes whatsoever except glycosuria. They may be feeling well and vigorous and be in a good state of nutrition. They may be overnourished. They may seek advice simply because they have been told that their urine shows sugar, and that they must seek advice. Others again may have, or may have had, certain symptoms of a type referable simply to an excess of sugar in the tissues, blood and urine, symptoms such as polyuria, thirst, fatigability, mental dulling, somnolence after meals, dimness of vision of the sort that results purely from changes in refraction, a balanitis, an intertrigo or the like. But these symptoms only occur as a rule when

the patient is indulging rather freely at table.

Also some of these patients may have symptoms of another type, referable directly to their arterial disease or to degenerative changes in the nervous system, symptoms that are not due directly to the well known metabolic anomaly that characterizes diabetes. We need not enumerate these symptoms, as everyone knows the group. It includes intermittent claudication, cramps in the muscles, gangrene, retinitis, loss of the knee jerks, perforating ulcers, polyneuritis, and so on. On the other hand, patients of the group to which we refer do not as a rule show the characteristic symptoms which result from an inability on the part of the body to *burn* enough glucose. They do not show severe acidosis or undernutrition except under certain special circumstances. It is of course true that an inherently mild case of diabetes in an older patient may be seen in a state of undernutrition, but if the case is in reality inherently mild the undernutrition will be due to the treatment imposed or to some factor other than the diabetes and not to the inability of the patient to use enough food to nourish him. It is also true that one may see one of these patients at times under the influence of an acute infection or under a nervous or emotional strain which is capable of temporarily depressing the glucose using power below the level that is characteristic for that case under ordinary conditions. The mild diabetes may then be fanned into a fury and the patient may develop a high acidosis. However, with proper management and the passing of the deleterious influence the case may re-

sume its former mildness, little the worse for the aggravation.

Cases of the type in question fall into two classes. There are those in which it is a very simple matter to make the urine sugar free and to keep it so without much annoyance to the patient and without reducing the diet to the point of weakening him. There are others in which in order to keep the urine sugar free the diet has to be depressed to a level so low as to impair strength and cause disability. Now when the disease itself is not disabling the patient and when in order to make the urine sugar free it is necessary to limit the diet so much that it will disable the patient in a week or two then it behooves us to pause and think. For if a doctor causes more disability in a week or two than the diabetes has caused in a year or two, he is 52 times worse than the diabetes from the patient's viewpoint. Before doing this he should be sure that what he will gain for the patient in the future by keeping the urine sugar free at the cost of immediate disability will repay what is certainly lost for him now, and before he undertakes the responsibility of disabling a man he must have very clear reasons for doing it. He will not do it on the basis of theory alone or of opinion unaccompanied by concrete evidence. Some patients have such confidence in the profession that they prefer to be disabled on a scientific regime rather than to be well and happy under a quack. But most patients are not so intelligent as this, and if the profession insists upon a disabling course of treatment unnecessarily they are prone to go outside of the profession for advice. I gain the impression that a

mistake sometimes made lies in a failure to discriminate between cases that can and should be kept sugar free and those which it is unnecessary or even harmful to manage in this way, and this raises the question of how we may discriminate between cases that should, and those that should not, be kept sugar free at all costs. With your permission I shall continue the discussion of this question by means of illustrative cases.

Case I.—The patient was a doctor 69 years old. He was 5 ft. 11¾ in. tall, and weighed on admission 154 lbs. The arteries showed marked alterations. The urine showed sugar, but no acetone bodies. Twelve years previously, at the age of 57, he was under some nervous stress and fell in body weight from 218 to 180 lbs. Examining his urine he found sugar, and he has had glycosuria practically all the time since, but it is not apparent that the loss of glucose calories in the urine was ever very great or enough to cause a loss of weight. In the beginning he could make the sugar disappear by adhering to a low diet, but the diet necessary to keep the urine sugar free was so low that it weakened him and he could not on that diet carry on his professional work. So he took more food and was able to work, although he then had some sugar in the urine. He lived the life of a diabetic from then on and always kept his diet as low as he practically could. During the following 11 years his weight remained in the neighborhood of 176 lbs. During the preceding year it had fallen to 154. He said that physically he was about 50 per cent efficient. He came in for insulin treatment, hoping that it might enable him to take more food and have more strength to work.

The following are the important points in the history:

(1) The glycosuria was discovered at 57. The great preponderance of all cases beginning at this age prove subsequently to be of a mild type, and from the age when the symptoms began we should rather expect *a priori* that the case would be mild.

(2) Since the beginning there had been no symptoms distinctive of diabetes except glycosuria. The loss of weight would be explainable with or without diabetes if the diet was as low as the patient said it was, and there was no positive evidence in the history to prove that the diabetes itself had caused undernutrition with the patient on a liberal diet. It is true that he lost weight before he dieted, but he was under a nervous strain and he may not have eaten his usual amount of food.

(3) The patient said he had felt better and stronger when, after desugarizing, he had taken more food, even though it caused a recurrence of glycosuria. He would probably not have reacted in this way unless he had been able to utilize some at least of the extra food that he took, suggesting that on the low diet he was not receiving all the food he was able to utilize.

(4) The diabetes existed 12 years in spite of the fact that during this time the urine contained sugar practically all of the time. No really severe diabetes could have continued so long as this under such conditions. Had the diabetes been severe it would long ago have declared itself in no mistakable terms. And this confirms what might have been expected in the first place.

(5) On admission the patient weighed 154 lbs. For a man 5 ft. 11¾ in. tall and large in frame this was decidedly a low weight. A year before and for the 10 preceding years he had weighed always in the neighborhood of 176 lbs., and the loss of weight during the previous year was not coincident with the appearance of new symptoms suggesting a severer diabetes. He did not develop polyuria, thirst or acidosis. But during the preceding year he was under a stricter dietary management and had taken less food, not because forced to do so physically by his diabetes, but because reading and reasoning and advice led him to take this action in order to forestall hypothetical future troubles that at this time existed only in his mind. Consequently we are inclined to discount the significance of this latest weight loss and to doubt that it could be due to the diabetes; for even if the diabetes had become more severe in the preceding year after having remained almost stationary for 11 years we should still be inclined to suspect that the aggravation would be temporary. Long standing cases of this

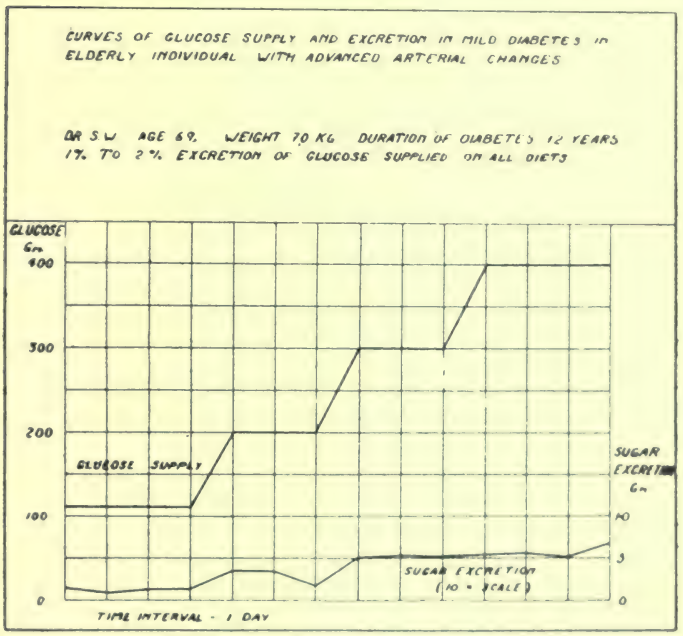
type may in the course of years gradually grow worse, but it would be very unusual to see one become suddenly and permanently more severe without a complicating factor after 10 years of station. At any rate on admission the patient was still able to hold a body weight of 154 lbs. or 70 kg. The body weight in kilograms times 30 gives 2100 as the number of calories required at light work to maintain this weight for a day, and the calories, divided by 17, gives roughly the least number of grams of glucose that must be burned by the average patient in order to produce the calories without using enough fat to cause acetonuria. So we know that this patient must have the power to burn some 123 grams of glucose at the minimum, and of course we suspect that he may be able to do much more. Furthermore, he is able to do this now after having had his diabetes for 12 years, during 11 of which he weighed always in the neighborhood of 176 lbs. and in spite of the fact that he showed sugar in the urine most of the time. Clearly he must have had and probably still has a high and durable sugar using power.

Starting with this knowledge, it might seem probable that if the patient were placed on a well balanced ration with a glucose value materially below 123 the urine would become sugar free, so he was placed on a trial diet with G at 110 grams as shown on the table. But the glycosuria did not wholly disappear or show any falling tendency in three days. Feeling convinced that he must be able to burn at least 123 grams of glucose and anticipating a much higher power still, we found that on a supply of but 110 grams he still excreted 1.0 gm. of sugar instead of 400 to 700 mg. as would be the case with a normal person (see Chart I). So on this diet the patient is using but 109 grams. This diet is so low that it will cause a further loss in weight and strength. The patient was undernourished and 50 per cent disabled when he entered. He came to get more food and strength, not less. He had asked for insulin, and in view of these findings one might be inclined to use insulin at this stage. But these findings would imply that the case was severe, for even a fairly severe case of diabetes may burn 109 grams of glucose, and this conflicts with everything in the history of this case, which had led us to conclude that the diabetes was mild.

In cases of this sort where there appear to be discrepancies between the laboratory data on the one hand and the clinical facts and common sense on the other, we have found it useful to place the patient on a series of diets with glucose values rising in steps of say, 25 to 50 or 100 grams. In this case the patient, having been placed for three days on the diet with G at 110, was then placed for three days on a diet with G at 200, and later on diets with G at 300 and 400 grams respectively in order to test the sugar using function on different supplies. When the excretion on a given diet becomes virtually constant (and in this case it did so at once on each of the diets given), the average for each of the several periods is plotted to give a curve. And by a study of the excretion curve we obtain valuable information. The curve is a curve of excretion, but the difference between the supply curve and the excretion curve is a curve of utilization. In this case it will be seen that as the glucose supply was increased stepwise from 110 to 400 grams the excretion rose but little, and that with each rate of supply almost exactly the same percentage of the supply was excreted, that is, about 4 per cent of 110, 4 per cent of 200, and so on. With this added information the discrepancy between the laboratory data and the clinical history comes to an end and the laboratory proves the case to be mild, as the history indicated it must be, for an individual who can on demand utilize 394 grams of glucose approaches the normal in respect of his sugar using power even if he does prefer to waste a little sugar in the urine. He must be able to produce daily the amount of insulin necessary for the utilization of as many grams of glucose as this. If we say, for instance, that 1 unit of insulin may be necessary in a given case for the oxidation of 2 grams of glucose, then this patient must be able to produce from his own islets some 200 units of insulin daily. (For without insulin glucose is not utilized, so far as we know.) So the patient, after 12 years of diabetes, 12 years of uncontrolled glycosuria, preserves this high insulin producing power. Yet in spite of it all, he shows an abnormal amount of sugar in the urine on any practical diet. On the low diet he is 50 per cent disabled. On a diet with a glucose value of 150 to 200 he is comfortable and strong, not disabled.

The blood sugar percentage is a trifle above the normal average on the higher diet, but also on the lower. It is about the same on both. He has no polyuria or thirst on either. He is 69 years old, and by his arteries older yet. What difference does it make to this elderly gentleman whether his urine when placed in a blue solution turns the blue solution red or leaves it blue? Surely he should not be disabled merely for that reason. He has no esthetic preference for blue as a color. As a matter of fact, as he says, in his earlier days he was inclined to favor the red. But after all, even if his urine is kept sugar free, will it make his calcified arteries young again?

But the gentleman came in for insulin treatment and was slightly disappointed not to have it. It might be thought that in a case of this kind it would require so little insulin to make the urine sugar free that there would be no reason why we


CURVES OF GLUCOSE SUPPLY AND EXCRETION IN MILD DIABETES IN ELDERLY INDIVIDUAL WITH ADVANCED ARTERIAL CHANGES
DR S W AGE 69. WEIGHT 70 KG DURATION OF DIABETES 12 YEARS
1% TO 2% EXCRETION OF GLUCOSE SUPPLIED ON ALL DIETS
GLUCOSE Gm
400
300
200
100
0
GLUCOSE SUPPLY
SUGAR EXCRETION Gm
40
3
0
SUGAR EXCRETION (10 = 1 GM)
TIME INTERVAL - 1 DAY

Case I Chart

should quibble over this detail. As a matter of fact, it happens very often in this type of case that a little insulin will not make the urine normally sugar free. In this case insulin was tried, as shown at the end of the chart. Twice as much was administered as might be necessary to remove the same number of grams of sugar from the urine of an ordinary severe case. But there was no lessening of the glycosuria. It so happened that the glycosuria actually ran a little higher on the insulin days. In most of our cases of this type we have found that insulin has had remarkably little effect per unit given and that the behavior in this respect may be characteristic and significant.

This patient was discharged on a diet with a glucose value of 160 to 180 that would easily yield all the calories needed for his comfort and permit of an increase in weight and strength. No further attempt was made to keep the urine sugar free, but to insure him against any possible error of judgment he was asked to submit monthly specimens for a year. I believe a large part of the trouble and disability endured by this

METABOLIC FINDINGS IN PATIENT WITH ADVANCED ARTERIAL CHANGES AND DIABETES OF LONG STANDING

OR S W

		DIET					URINE					
								SUGAR				
DATE	KG. WEIGHT	C	P	F	G	CAL.	VOLUME	DAILY	AVERAGE	N	N×6.25	REMARKS
10-18	71.0	64	53	140	110	1728	2040	1.17				Bl. Sug.-0.13
19		"	"	"	"	"	1230	0.78		9.4	58.7	
20	68.6	"	"	"	"	"	1230	1.03				
21		"	"	"	"	"	2100	1.07	1.01			Excr. 1.0% of Supply
22		154	55	137	200	2066	1400	3.55		6.1		
23		"	"	"	"	"	1300	3.44				Excr. 1.4% of Supply
24	70.0	"	"	"	"	"	1000	1.63	2.88	8.5	45.6	Bl. Sug.-0.15
25		256	59	86	300	2034	1900	5.07		5.5		
26		"	"	"	"	"	2400	5.20		8.3		Excr. 1.7% of Supply
27	69.0	"	"	"	"	"	1660	5.10	5.12	8.1	45.6	Bl. Sug.-0.15
28		356	59	86	400	2434	2440	+				Dextro-rotatory
29		"	"	"	"	"	2300	5.72		6.3		Excr. 1.4% of Supply
30		"	"	"	"	"	1700	5.32	5.52	8.3	45.6	Bl. Sug.-0.13
31	70.0	"	"	"	"	"	1880	6.89		7.9		Bl. Sug. 11 A.M.-0.15 Insulin 10 Units

Case I Table

patient in the 12 preceding years was unnecessary. Had he succeeded in keeping the urine sugar free, or had he lessened his body weight more, his good tolerance in the end might have been interpreted as a triumph of treatment, but he failed to keep his urine sugar free and for 11 years he weighed 176 pounds, all of which would seem to prove that the disease itself was inherently mild and non-progressive throughout.

Case II.—In contrast with the excretion curve contained in this case is the curve obtained in a case of genuinely severe diabetes in a woman of 31 shown in Chart 2. This curve is typical of those obtainable in cases of severe diabetes in young people.

The lower serrated line shows the total excretion of sugar by days as measured in milligrams by the Benedict-Osterberg method. The upper

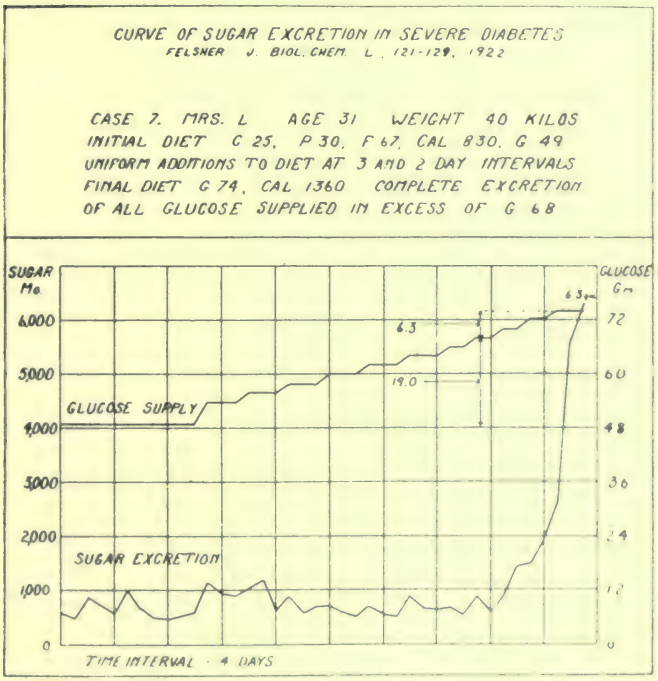


Chart 2

terraced line shows the glucose value of the diet ascending stepwise at intervals of three days. In this instance each step represented an addition to the diet of a small uniform quantity of a milk-cream mixture that increased each time the glucose value of the diet by 5 grams. On the initial diet level the urine was sugar free to the qualitative tests with daily excretions below 1000 mg. Each increase of the diet caused the urinary sugar for the first day to rise a trifle, but the initial rise was followed always by a return to the former level on the days following. It will be noticed that as the G of the diet was worked upward 19 grams there was no permanent rise in the output of sugar. On the contrary, it actually settled to a level somewhat below the initial level. Then with one of the regular additions to the diet came an increase that was not followed by a restoration of levels on the following day, a warning. The next addition shot the excretion upward and was almost quantitatively excreted. This chart is one of those that appeared in Miss Felsher's report on tolerance limits in severe diabetes. It will be noticed that in this case the excretion and utilization appear to be normal up to a certain level. The insulin supply is capable of rising with the glucose supply as in health up to a certain point. But apparently that is nearly as high as it can go. The patient uses up to 65 grams of glucose with normal completeness. If more is given little if any more is used, a very different phenomenon from that shown by Case I, who used more and more glucose the more he was given (up to 400 grams).

Case III.—Chart 3 shows a curve obtained in the case of an elderly woman who it was thought might possibly prove to be a case of the same type as Case I, but it will be noted that the sugar excretion was normal with the G of the diet below 120 and that the curve broke rapidly upward when the supply exceeded 120. The behavior was like that of Case II and unlike that of Case I. Although this case could easily be held sugar free without insulin, she was a working woman and was partly disabled on the lower diet, so her food was increased somewhat with the assistance of insulin to give her a diet with a "G" of 140.

Cases IV and V.—Charts 4 and 5 show curves

obtained in a series of cases of the type which is commonly called renal diabetes or renal glycosuria. They closely resemble the curve of the first case, but all of these latter curves were obtained with younger people. These cases also show no effect from insulin. The blood sugar percentages were low, differing in this respect from the type of case illustrated by Case I. Some years ago S. Strouse, of Chicago, pointed out that with his cases of renal glycosuria gross changes in the magnitude of the diet caused relatively little change in the glycosuria, much less in fact than would be shown by cases of typical diabetes. He emphasized this as a characteristic feature of these cases. The curves presented illustrate this point very clearly, and give the phenomenon a quantitative expression.

COMMENTS AND INTERPRETATION

The cases described in the foregoing are all of known types. The disease presented by Case I has been described in the literature, but is not so often recognized as the others, and the differential diagnosis has been less clearly established. It is representative of a group in which there is a close resemblance to the so-called renal diabetes or renal glycosuria, but in which the blood sugar percentages run above the normal average, rather than below it. In some of our cases of this group the blood sugar percentages have run much higher than those recorded in Case I. The report illustrates a method of study, a procedure in some respects new. The method consists in subjecting the body to a series of glucose supplies, rising or falling as a straight line, the individual being kept on each of a succession of diets for three or more days, during which the 24-hour outputs of sugar are measured by the Benedict-Osterberg or Folin-Berglund method for the estimation of sugar in normal urines. When the excretion becomes

virtually constant for any one rate of supply, the average is taken as the excretion for that rate, and so on with each of the diets in succession. The results are then plotted in the form of a curve. The curves so obtained are curves of excretion, but the difference between the excretion curve and the straight line supply curve give a curve of utilization and we are then enabled to study the rate of utilization as affected by supplies ranging from relatively low to relatively high levels or the reverse. During such a test there is of course no objection to plotting also the changes of the blood sugar percentage. But it may be pointed out that the total excretion for 24 hours when measured in milligrams by a method such as one of those mentioned, represents the aggregate effect for the whole period, and that during this period ups and downs of the blood sugar percentage and of the excretion occur. To obtain an aggregate picture of the blood sugar percentage requires multiple punctures and determinations at intervals all through the day at the cost of discomfort to the patient and time and labor for the observer. Moreover the results when obtained do not yield more and as a rule less direct information than that given by the excretion. The blood sugar curve does not indicate absolutely the number of grams of sugar excreted and utilized respectively, the point of primary importance.

From the viewpoint of physiology, the procedure described has the advantage of permitting one to sustain a given supply rate for a long enough time to reduce to a minimum those errors that arise from periods of observation that are too short to

METABOLIC FINDINGS IN CASE OF MODERATELY SEVERE DIABETES WITH ARTERIAL CHANGES

MRS. D.M

DATE	KG WEIGHT	DIET C	P	F	"G"	CAL.	URINE SUGAR	REMARKS
9-23 to 27	52 6	20	33	105	50	1157	0 77	Bl. Sug · 0.22
10-6 to 8	52 8	58	50	135	100	1647	0 77	
9-7 to 11	52 7	74	50	165	120	1981	2 68	Bl Sug 0.26
9-12 to 22	53 8	90	58	202	150	2450	12 67	Bl. Sug · 0.31
9-23 to 28	54 0	149	58	178	200	2562	34 80	Bl. Sug. · 0.37
9-29 to 30	54 0	198	59	178	250	2562	60 80	

SUMMARIZED MANAGEMENT OF CASE

9-30 to 10-5	52 6	20	33	105	50	1157	0 79	
10-6 to 8	52 9	58	50	135	100	1647	0 77	
10-9 to 11	52 4	71	59	155	117	1901	1 50	
10-12 to 18	54 4	·	·	·	·	·	0 56	Insulin 10 U.
10-19 to 24	54 4	81	54	179	133	2081	0 59	· ·
10-25 to 28	55 0	84	57	188	141	2297	0 87	· ·

FURTHER STUDY OF DATA OBTAINED

SUPPLY "G"	EXCRETION	UTILIZATION "T"	SUPPLY INCREMENT	EXCRETION INCREMENT	% OF "G" INCREMENT EXCRETED
50	0	50			
100	0	100	50	0	0
120	3	117	20	3	15%
150	13	137	30	10	33%
200	35	165	50	22	44%
250	61	189	50	26	52%

CURVES OF DATA OBTAINED

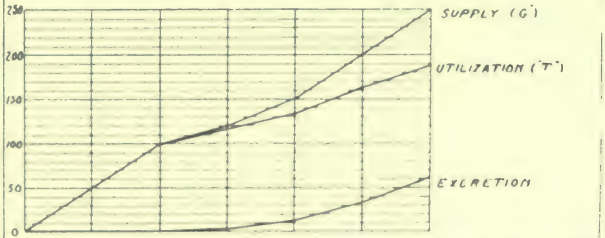


Chart 3

allow the body to adjust itself to the conditions. It not infrequently happens that following the initiation of a specified rate of supply the permanent effects of that rate will not be dependably registered in the first hour or day, either in the urine or blood.

The clinical experiments described show that when different types of cases are studied as described they yield two distinct types of excretion (and utilization) curves. The method reveals a difference in the behavior of different types of cases. This appears to be a fact. That cases may be found which yield mixed or transitional types of curves might be anticipated. The question rises as to what these different types of curve mean, physiologically and clinically, and in this connection it is unnecessary to urge any fixed ideas at the present time. Certain possibilities may be suggested, however.

It would seem to be definitely established that for the utilization of glucose in the body insulin is necessary, although of course insulin is not all that is necessary. Suitable intracellular conditions are required in order that insulin may exercise its function, and other conditions might be enumerated. But insulin is the *sine qua non*, and without it there appears to be no considerable oxidation of glucose. When therefore in an individual such as Case I it is possible to demonstrate that with a glucose supply as high as 400 grams per day, all but 4 to 6 grams are utilized day after day, the individual must be able to produce enough insulin to effect the utilization of 394 to 396 grams of glucose. At the empirical figure of 1 unit of insulin to 2 grams of sugar this would

imply the power to produce at least 197 units of insulin per day, and whatever else might be wrong with the patient's body it would seem certain that he did not lack insulin producing cells in quantity sufficient to produce this large amount of insulin when called on to do so. We have strong reason to believe that a large percentage of all the insulin made in the body arises from the cells of the islets of the pancreas, because if they are totally removed the utilization of glucose sinks to a very low level. In cases of "total" diabetes in man 50 grams of glucose given by mouth may be recovered almost quantitatively in the urine in properly conducted experiments. Total pancreatectomy in dogs lowers the glucose utilization less perhaps, but still very greatly. The curve obtained in Case I might enable us to say, therefore, that whatever else might be amiss with the individual he possessed enough functionally active islet epithelium for all ordinary purposes. This conclusion would seem to be strengthened by the fact that the glycosuria was not reduced when the patient was given additional insulin from an outside source, enough in fact to eliminate a greater glycosuria in another type of case. Could it not be said then that this individual had no hypo-isletism in the sense of an underproduction of insulin by the islets of Langerhans? Does it not suggest that in this case the reception of insulin in the tissues or at least the effective employment of insulin in the tissues or the cells might be responsible for the glycosuria?

Insulin has been observed to be less effective per unit injected when administered to patients in a state of acidosis

DIET, URINARY DATA, ETC., IN CASE OF SO-CALLED "RENAL GLYCOSURIA"

Date	DIET C	P	F	"G"	Cal.	URINE Volume	Sugar Gm.	Blood Sugar %	REMARKS
10–10 11	324	100	175	400	3271	1680	4.0		
10–11 12	324	100	175	400	3271	1440	3.0		
10–12 13	324	100	175	400	3271	1780	2.8	0.07%	
10–13 14	324	100	175	400	3271	1540	3.1		Excretion=1% of Supply
10–14 15	140	80	138	200	2122	1000	1.0		
10–15 16	140	80	138	200	2122	1500	1.8		
10–16 17	140	80	138	200	2122	1020	2.0	0.07%	Excretion=1% of Supply
10–17 18	50	62	144	100	1744	1240	0.9		
10–18 19	50	62	144	100	1744	1140	0.9	0.06%	Excretion=1% of Supply

CHART 4

DIET, URINARY DATA, ETC., IN CASE OF SO-CALLED "RENAL GLYCOSURIA"

Date	DIET C	P	F	"G"	Cal.	URINE Volume	Sugar Gm.	N	REMARKS
2–11									
12	47	70	128	100	1620	2200	5.7		Blood Sugar 0.10%
2–12									
13	47	70	128	100	1620	1560	5.5		5–6% of supply excreted
2–13									
14	47	70	128	100	1620	3300	4.6	7.0	
2–14									After fermentation
15	47	70	128	100	1620	2040	6.7		Urinary sugar=0.696 gm.
2–15									
16	140	80	138	200	2122	2400	16.0		Dextro-rotatory
2–16									
17	140	80	138	200	2122	1700	12.8		6–8% of supply excreted
2–17									
18	140	80	138	200	2122	2160	18.5		Blood Sugar=0.10%
2–18									
19	140	80	138	200	2122	1360	11.2	8.5	Insulin (Iletin No. 753) 5 units
2–19									
20	140	80	138	200	2122	2500	12.4		Insulin (Iletin No. 753) 10 units

CHART 5

(with an alkali deficit) than it is in the same patient in the non-acidosic state. This I can vouch for from observation on a case of long standing virtually complete diabetes in periods in which acidosis was present and absent respectively. Infectious processes, as all know, may reduce the effectiveness of administered insulin quite apart from their power to depress endogenous insulin production. From such observations we know that a sufficient quantity of insulin may be supplied and still prove only partly effective. It might seem quite possible that in cases like Case I the tissue or cellular condition makes insulin less effective. The arterial disease might possibly be identified with these tissue conditions. Possibly in such a case we are dealing with a mottled or spotty diabetes, if we may so describe it, a peripheral or tissue diabetes, as opposed to a general or central diabetes of islet origin. Some of the tissues or cells of the body might be conceived as burning glucose well, others less well. The latter excreting their unburned sugar into the blood would account for an hyperglycemia, with some overflow into the urine.

Another idea that might be stated for what it is worth is that possibly the islets in such a case, although capable of high insulin production under stimulation are relatively sluggish in their responses to normal stimuli. The innervation of the islets or the blood vessels of the islets might be damaged, possibly as part of the same general process that affects the arteries and nerves of the feet and legs in these same cases. If this were true there would be in such cases a relative lack of insulin for any given supply of glucose.

The blood sugar percentage would have to rise to a higher level than in a normal case to stimulate the islets to the same production of insulin. This idea might seem less tenable than the other on the ground that if this were the case one might rather expect a greater result from administered insulin, although this would not positively negative the idea, since the administration of insulin by causing a beginning fall in the blood sugar percentage might induce a compensatory lowering of the endogenous insulin supply and so have no permanent effect on the amount of insulin in circulation.

Cases which give curves like that shown in Case II or any curve in which increasing the glucose supply up to a certain level causes relatively little or no rise in the excretion, but in which progressively increasing the glucose supply beyond this point causes a rapid and progressive increase of the percentage of the supply excreted, as shown by an upward bend or break in the curve, suggest a definite inability to produce insulin in excess of a more or less sharply definable limit. The possibility is suggested that this type of curve indicates a deficient insulin production, a deficiency of the islet apparatus itself, a general diabetes of central or islet origin. One might perhaps picture islets capable of functioning normally under limited loads but failing like weakened hearts when called upon to do more.

The group of cases classified as cases of renal glycosuria show curves resembling that of Case I and would indicate also that the insulin production in general is not

greatly subnormal. Whether these cases are also due to conditions in the tissues and whether they represent a special group of what we have alluded to as peripheral, or tissue diabetes with the kidneys involved in the process, is a conjecture, but one not without possible interest. Whatever interpretation may be preferred, the study of excretion and utilization curves under a known range of glucose supplies presents, I believe, a method of testing the sugar using function which brings out new possibilities.

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LEUCOPLASTS: LIVING, REPRODUCING, PERFECT CHEMICAL CATALYSTS*

BY EDGAR J. WITZEMANN

It is the purpose of this paper to visualize in chemical terms the familiar operations of leucoplasts as perfect living chemical catalysts. It is hoped, by restating the familiar in these physical chemical terms, that the viewpoint developed may constitute a preparation for a similar visualization of other chemical catalyses in living cells. This paper is also intended to be a contribution to the study of the transition from crystalloid to colloid properties previously discussed in broader terms[1].

(1). *Introduction.* Among the enzymes best known chemically are those that are normally secreted into the gastro-intestinal tract. The conditions by which the activities of these enzymes are modified are pretty well known, so far as the hydrolytic phase of their activities is concerned. On the contrary little is known about the reverse effect—namely condensation—as exercised by these enzymes in conditions that may be considered normal for life[2]. More is known about the synthesis of esters by lipase than any of the condensation reactions here involved[3]. This may be due to the fact that the simpler chemical constitution of fatty acids renders them more immune to slightly unfavorable conditions than the sugars and amino acids, for instance. But whatever the reason, it is well known that these enzymes do not reverse the reaction to the point of equilibrium. They are therefore lacking in this important characteristic of a perfect chemical catalyst. One of the simplest possible explanations of this fact is the idea that chemical investigation has not yet surrounded these hydrolytic enzymes with the proper conditions, in order to enable them to synthesize their substrats as efficiently as they hydrolyse them. This suggestion is also supported by the fact that synthetic reactions are carried out almost entirely within the cells by enzymes *in situ*. The question at once arises as to whether we can view the chemical operations of any enzyme *in situ* with sufficient accuracy to determine whether it is a more perfect chemical catalyst under these conditions than in *in vitro* conditions. This paper constitutes a review of the best known chemical and biological facts concerning the synthesis and hydrolysis of starch in the living plant cell. The results clearly suggest that the enzyme functioning *in situ*, in this case, is more perfect in the accepted physical chemical sense than the common enzyme preparations with which we normally work.

(2). *How is starch formed?*[4] The commonest, readily hydrolysed, polysaccharides are the starch of plants and the glycogen most commonly found in animals. They differ most strikingly, however, in that the latter is generally

*Contribution from Otho S. A. Sprague Memorial Institute, Rush Medical College, Chicago, Ill.

an invisible jelly while the former is made up of visible sphero-crystals. The formation of the former has been much studied and is easily observed in comparison with the formation of the more gelatinous glycogen in animal tissues. In plants starch appears in two visible relations. It appears temporarily as very small grains in protoplasm and in chloroplasts whenever sugar accumulates locally and it appears as larger more permanent starch grains in connection with those other protoplasmic corpuscles known as leucoplasts. The young starch grain generally appears in the center of the leucoplast as a spherocrystal. Later the leucoplast often tends to become thicker on one side and the crystals assume various shapes depending on the circumstances. The situation may be graphically stated thus: The leucoplast is lying in a dilute solution of sugar while in its interior it is laying down (secreting)[5] a stable condensation product—starch—and is producing thereby a relatively enormous concentration of inert sugar locally. In chemical terms the leucoplast is a catalyst for condensation which has the power of selectively removing sugar from dilute solutions. There is nothing unusual in finding a system acting as a catalyst one of whose properties is the capacity to raise the concentration of a component of the substrate upon its surface or within its boundaries. The action of the leucoplast in this respect therefore coincides with what the colloid chemist calls adsorption. This adsorption like that of the colloid chemist may be positive or negative depending on the concentration of the sugar solution with which the leucoplast is in equilibrium. When the sugar concentration outside the leucoplast falls through a slight range to a point below a certain critical level the adsorption becomes negative in sign and the starch grains begin to undergo erosion. The main difference between the classical adsorption of chemistry and that of the leucoplast is that the condensation brought about by the latter is visible as starch, while in adsorption phenomena in general the fact that condensation or concentration occurs must be deduced mathematically[6].

The leucoplast thus constitutes an ideal chemical catalyst in that it catalyses the reaction in both directions depending upon changes in the environmental conditions one of which is a slight change in the concentration of the sugar solution in which it lies. In this sense it may also be considered to be an ideal enzyme for sugar condensation such as has apparently never been isolated or tested under suitable conditions. Moreover since the leucoplast is apparently a somewhat differentiated portion of the general protoplasm[7] we can identify this enzymatic or catalytic process as a chemical function of protoplasm. This identification is also supported by the fact that small granules of starch are said to be formed throughout the protoplasm of the cell upon slight provocation. These granules are more readily dissolved apparently than those formed in the leucoplasts, so that there appears to be some differentiation in this respect also, although this may be due merely to the well known greater solubility or solution tension of small crystals in the presence of larger ones.

3. *Glycogen formation.* Glycogen differs most strikingly from starch in being less sharply differentiated *in situ* in the cell[8], in being hydrolysed with great rapidity after the death of the organism, and is therefore in all ways more difficult of access. Like starch in plants, glycogen is present to some extent in all parts of the animal organism. The greatest concentration occurs in the liver so that this organ in a sense takes the place of seeds, fleshy tubers, corms, bulbs, etc., of plants as a special storehouse for reserve carbohydrates. The cell "organs" in animals that correspond to the leucoplasts in plants must be very numerous and efficient in the liver. These glycogen-forming elements in the liver have perhaps never been described, but their existence is amply provided for by the complete analogy in all the relations as to constitution, properties, distribution, mobilization, etc., of starch and glycogen.

4. *The role of organization in chemical catalysis.* It may be objected by some that in considering the leucoplast as a catalyst we have moved the problem of condensation in living organisms out of the field of chemistry. This is not true. We have merely acknowledged that a suitable physical state of organization and a suitable chemical environment is more necessary to this catalytic process than is commonly realized. In this respect it then resembles many well-known chemical catalysts which we have become thoroughly accustomed to "petting" in order to sustain their optimal activity. The role of organization in oxidizing catalysts was pointed out by Warburg[9] and by Battelli and Stern[10] and is now generally accepted. Is there any good reason for believing that the unknown oxidizing enzymes and catalysts in general operate on different principles from those concerned with condensation and therefore that one can subject them to any kind of treatment and expect them to retain their activity?

This conception of organization in living organisms involves a suitable physical condition and generally a suitable admixture of other substances. These two conditions must also be met in chemical catalysis. Thus for instance the best catalyst for converting CO into CO_2 at ordinary temperatures is a mixture of four oxides[11], which, for unknown reasons, works better than any of the components taken alone. The fact that a leucoplast is an organized mixture of substances in a suitable condition can therefore not be used as an argument against its being a chemical catalyst.

5. *What makes the condensation reversible?* We have pointed out above how the larger starch grains are found in leucoplasts while those occurring in the general protoplasm are usually smaller. We have noted how rapidly they are formed or disintegrated depending on conditions. We have also pointed out the fact that the variations in concentration of the sugar in the cell sap are sufficient, in terms of the little that we know about adsorption, to account for the change in sign of the adsorption (negative $\rightleftarrows$ positive) with fluctuations in the sugar concentrations. On the other hand it is well known that other components of the medium vary in concentration fully as much as the sugar does, that variations in the concentration of other substances modify the velocity of catalytic processes and that sugar is certainly not the only sub-

stance that can be differentially adsorbed by a leucoplast or by protoplasm in general. Thus it is well known that H^+ ions favor hydrolysis and OH^- ions are unfavorable to the hydrolysis of starch. If the concentration of these ions or their biological analogs varies in the medium then they will be adsorbed by the leucoplast in variable amounts and give rise to a secondary acceleration or retardation of the corresponding process in question. It is in this way and at this point that the presence or absence of internal secretions, etc., probably acts to inhibit or accelerate the hydrolysis or the formation of glycogen in animals. The variations of the concentration of these compounds in the medium gives rise to secondary variations in particular zones of chemical action depending on the adsorption relations of the phases in question to the chemical substances involved.

These accessory agencies do not complicate the chemical picture of the simple underlying chemical processes. They take up the "slack" or "lost motion" of the finer adjustments of the equilibrium and provide a mechanism for more readily maintaining the equilibrium concentrations whenever they are disturbed. To attempt to discuss them in detail now, even if we were prepared to do so, would merely help us lose sight of the simplicity of the fundamental catalytic process.

6. *The condensation hydrolysis equilibrium involved in the formation and mobilization of starch.* The central point of interest in the chemical mystery of the formation of starch is the actual formation and deposition of the polymer starch. Plant physiologists have demonstrated repeatedly that a leaf that has been completely freed from starch by etiolation synthesizes enough starch from CO_2 or sugar solutions in a few minutes after exposure to sunlight to give a positive iodine test for starch. In the hands of the chemist glucose can be slowly converted into polysaccharides only with the greatest difficulty[12]. There are in this respect two types of substances (1) those like water that polymerize and depolymerize freely and spontaneously, and (2) those like sugars that do not. If a substance naturally belongs to the second class there is very little that the chemist can do at present to move it into the first class, because the general subject of the catalysis of condensation equilibria is still wrapped in darkness. One interesting thing however stands out clearly: in plants, and probably in animals, glucose belongs to the first class. If we attempt to explain this difference in the two environments we presently hit upon the idea that perhaps substances of the second class require "preparation" for condensation such as is supplied by insulin in animals in the metabolism of glucose. The most effective agencies for catalysing chemical condensations *in vitro* are weak alkalies like ammonia, pyridine and alkali compounds in general. These are generally thought to give rise to a certain amount of highly reactive isomers, such as the enol forms of certain ketone derivatives, which then condense spontaneously.

Without attempting to review the present unsatisfactory state of this problem in organic chemistry it is even now clear in about what direction the

solution of this mystery of spontaneous condensation is to be sought, although the answer can not be concretely discussed or demonstrated *in vitro* as yet.

7. *How are leucoplasts formed?*[13] In considering leucoplasts chemically we ought if possible know how they are made.

Botanists generally believe that most starch is produced by leucoplasts (which they consider to be chloroplasts without chlorophyll), which are multiplied and transmitted from cell to cell by division and are not formed *de novo*[14]. The more recent view is that they arise by a demonstrable differentiation of "mitrochondria" (chondriosomes) which in turn have been observed in all the cells of the plants studied at all stages of development (even in pollen grains)[15].

The chemical differentiation involved is probably not great because it can only be demonstrated by the most recent and painstaking histochemical technique. But if either of these views is correct the formation of leucoplasts is chemically simple.

The existing leucoplasts simply grow by the addition of material until they become large enough to break up or divide due to the fundamental causes that make any particles in an emulsion break up. It is not hard to add material to one phase in a system without appreciably changing the composition of another phase. Or in other words a given phase may grow in size and change in composition without appreciable growth or change in any other phase in contact and in equilibrium with it. Thus if we add oil to an ether solution of oil-in-water suspension we find, when the phases separate so that we can measure their volumes, that the ether layer has been increased. Very little oil dissolved in the water. If water is added instead of oil the results are equally simple but reversed. Moreover our illustration shows us that the phase that is increased is determined by the solubility relations of the component offered to the system. Leucoplasts can and no doubt do have a selective adsorption for some of the compounds of which they are built up (as well as for sugar) that will be great enough to cause them to grow in a true physiological sense[16]. There is abundant chemical experience with systems that behave in the same physical ways that living systems such as leucoplasts do. Moreover the outward similarity persists down to the observable limits of small objects. But no such systems however similar they are outwardly carry the physiological traits of life. Our interest in these analogies is great however because they give us a clear picture of how our living enzyme or leucoplast can function, grow and multiply without requiring us to believe that it does so by virtue of a chemical behavior entirely unknown to us.

The botanist's conviction that leucoplasts increase by division and that they are passed on from cell to cell practically in a preformed condition simplifies the whole problem as to their origin and existence in cells. The problem of increasing their number and activity then becomes one of suitable nutrition of those already present. Considerable progress has been and is being made in the study of enzymes by studying the nutritional factors that are associated with their minimal or maximal activity.

If on the other hand we accept the dissenting view, for which there is also considerable support[17] that although leucoplasts divide, they may also arise *de novo* in the cytoplasm, the chemical conditions of their formation are materially and favorably changed. Under the former view promotion of development of leucoplasts by nutrition is hopeless if there are no leucoplasts present in the cell which can undergo division. Under the latter view it should be possible to promote their formation *de novo* by suitable nutritional adjustments in cases in which they were entirely absent from some of the cells.

8. *Application to diabetes.* Aside from its interest in giving another clear view in the chemical study of enzymes the above discussion of the chemical nature of leucoplasts is of interest in the study of diabetes. The analogies of the formation of glycogen and of starch are, as was stated above, close. It has recently been found that the formation of glycogen can be restored in diabetes by the administration of insulin and it is therefore possible that insulin is an indispensable component of the "glycogen leucoplast" system. In fact the recent isolation of insulin-like materials from yeast[18], from various other plants, from animals and from animal organs suggests that it may be a fundamental component of protoplasm and perhaps a component of true leucoplasts. The available facts concerning insulin indicate that it resembles enzymes in a general way. It is known that enzyme formation and enzyme activity are modified by nutrition. These and other facts, that we need not stop to enumerate, raise the important question as to whether the growth and development of the various catalysts or enzymes involved in diabetes can be improved or restored, by suitable nutritional considerations. There are enough indications that this question is capable of an affirmative answer to justify a long search, by those who have the experimental material in hand, for the definitive factors involved.

9. *Energy relations.* After having made a qualitative survey of the problem of starch and glycogen formation it is well to check up on the energy relations. As soon as we turn to consider the problem in such terms it becomes almost glaringly obvious that the processes of the leucoplast as pictured in this paper show a beautiful conformity to the Law of Supply and Demand otherwise known in chemistry as LeChatelier's Principle. This interesting generalization is stated by Lewis[19] as follows: "When a factor determining the equilibrium of the system is altered the system tends to change in such a way as to oppose and partially annul the alteration in the factor". This law is a qualitative statement of the Second Law of Thermodynamics and as such is probably the most widely applicable generalization known concerning natural phenomena[20].

The most obvious factor involved in the phenomenon of condensation and hydrolysis here under discussion is the close relationship between the ebb and flow of sugar into the leucoplast and the supply and demand of sugar from other parts of the plant. There is here a spontaneous natural equilibrium between kinetic energy just stored in the form of potential energy as newly formed sugar, a less labile type of potential energy as starch and a more labile

form of potential energy as mobilized starch. Probably the newly formed sugar and mobilized starch are identical isomers of glucose. In other words we have an equilibrium involving two forms of potential energy obviously controlled by the Second Law of Thermodynamics, which is strikingly visualized by the supply and demand concept. This principle of the chemical philosophy of plants may be graphically represented thus:

Chemically labile, physically stable but diffusible, dialysable glucose. $\rightleftharpoons$ Physically labile, chemically stable, non-diffusible, non-dialysable starch.

This equilibrium also visualizes an interesting differentiation of energy which was more fully discussed in general terms in a previous paper[21]. If we consider glucose and starch as two closely related forms of potential energy we have in the low glucose concentration of the cell plasma an equally low intensity of energy concentration, while with starch we have a highly concentrated form of glucose with a corresponding high intensity of potential energy. Since hydrolysis is said to be an exothermic reaction energy used up in the "work" of condensation must be derived from some other source. In the case of the leucoplast this may arise from the oxidation of part of the sugar.

The tremendous simplicity and efficiency of the energy relations involved here and the perfect "team work" with which the leucoplast "plays up" to the chloroplast become more overpowering the longer one considers them. It sometimes seems that if anything in the world is a more perfect physical chemical mechanism than one of these it is undoubtedly the other.

Summary

By considering the leucoplast as the enzyme which catalyses the starch-sugar equilibrium we clarify the whole problem of enzymatic condensation for ourselves and escape a number of difficulties.

(1) Our enzyme is a perfect catalyst in a physical chemical sense.

(2) It functions as a segregated corpuscle which is visible in the living cell and can not therefore be an artifact arising from post-mortem changes.

(3) It represents a differentiated function that is not so efficiently performed by protoplasm as a whole.

(4) It multiplies within the cell by division (generally).

(5) Its efficiency is no doubt due to its chemical constitution and its physical state of organization. Press juice containing leucoplast juice and other material more uniformly mixed could not *a priori* be expected to function well in the formation of starch though it might hydrolyse polysaccharides rather well.

(6) In this respect the leucoplast resembles the better known chemical catalysts (like platinum) which are highly active under suitable conditions but not so active or even entirely inactive under other conditions.

(7) It is also interesting to note that the problem of condensation when viewed in its structural relations in this way parallels the observations of Warburg on the role of structural organization in vital oxidation.

In the later sections of the paper the possible existence of glycogen forming leucoplasts is briefly considered on the basis of analogy. The existing viewpoints on the origin of leucoplasts are further extended in order to discuss their formation in the cell. From any of the existing viewpoints as to their formation the problem of the primary formation and multiplication of such organized catalysts is for the present still a problem in nutrition.

The qualitative relations developed conform in interesting ways with the qualitative statement of the Second Law of Thermodynamics known as Le Chatelier's Principle. Furthermore the glucose $\rightleftarrows$ starch equilibrium involves an interesting differentiation of potential energy.

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[1] E. J. Witzemann: J. Phys. Chem. **26**, 201 (1912).

[2] We know that condensation is quantitatively one of the most abundant as well as one of the most delicately adjusted types of reactions that take place in the living organism. All higher life is organized on that basis. Condensation is the reaction that enables life to persist during periods of unfavorable fluctuations in the food supply. The facts are glaringly obvious from any point of view and yet we can not interpret them chemically because we know little or nothing about condensation, even in chemistry.

[3] Cf. W. M. Bayliss: "The Nature of Enzyme Action". 3rd Ed. Chapter on reversibility of enzymes for other examples and fuller discussion.

[4] The botanical facts here given will be found in J. Reynolds Green's "Vegetable Physiology," p. 224, 2nd Ed. (1907) or in F. Czapek's "Biochemie der Pflanzen," I, 397-417 (1913). A more extended review of the subject of starch is given in E. T. Reichert's Monograph: "The Differentiation and Specificity of Starches in Relation to Genera, Species, etc. Carnegie Institution of Washington, Publication No. **173**, pp. 17-163 (1913).

[5] J. Reynolds Green: "Vegetable Physiology," p. 228, 2nd Ed. (1907).

[6] W. D. Bancroft: "Applied Colloid Chemistry," p. 24, for example.

[7] Cf. especially A. Guilliermond: Arch. Anat. Micro. **14**, 309-428 (1913) on the formation of leucoplasts by differentiation of mitochondria (Chondriosomes).

[8] J. Reynolds Green: "Vegetable Physiology," p. 232. Glycogen in fungi sometimes separates as granules, "which to a certain extent resemble grains of starch and which have been stated to originate in certain corpuscular bodies resembling leucoplasts. In most cases the deposition appears to be effected by the protoplasm".

[9] O. Warburg: Erg. der Physiol. **14**, (1914).

[10] Biochem. Z. **67**, 443 (1914).

[11] A. B. Lamb, W. C. Bray, and J. C. W. Frazer: J. Ind. Chem. Eng. **12**, 213 (1920).

[12] J. U. Nef: Ann. **403**, 223 (1914).

[13] See Lester W. Sharp: "An Introduction to Cytology," pp. 104, 106, 109, 113, 121, 122, (1921) for a summary of the present status of the various views on the nature and origin of leucoplasts.

[14] "There are some other small bodies which are free from coloring matter, and which form starch from sugar. We call all these protoplasmatic organs which are in the service of carbohydrate metabolism, *Plastids*. As far as we know, they are never formed from other plasmatic parts. They always take their origin from mother plastids by cleavage". F. Czapek: "Chemical Phenomena in Life," p. 59.

[15] Cf. Guilliermond: *Loc. cit.*

[16] The capacity of growth of leucoplasts presupposes a selective adsorption by leucoplasts of the components of which they are synthesized . This however represents the transition from crystalloid to colloid properties within homologous series previously discussed. Without attempting to discuss the case in detail we have here an apparently spontaneous transition from the crystalloid state to the colloid state of the components of leucoplasts for the development of a catalyst (the leucoplast), adapted to enormously facilitate the transition of crystalloid sugar into colloidal starch and *vice-versa*. So far as present knowledge goes the formation of the leucoplast is an autocatalysis.

[17] Cf. Sharp: Loc. cit. p. 118.

[18] L. B. Winter, and W. Smith. (Proc. Physiol. Soc.) *J. Physiol.* **57**, XL (1923).

[19] Wm. C. McC. Lewis: "A System of Physical Chemistry," Vol. II, p. 109, 3rd Ed. (1920).

[20] Cf. W. D. Bancroft: J. Am. Chem. Soc. **33**, 91, (1911) and W. C. McC. Lewis: Loc. cit.

[21] E. J. Witzemann: J. Phys. Chem. **26**, 201 (1922).

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[CONTRIBUTION FROM THE OTHO S. A. SPRAGUE MEMORIAL INSTITUTE, LABORATORY OF CLINICAL RESEARCH, RUSH MEDICAL COLLEGE]

THE ACTION OF GUANIDINE UPON GLUCOSE IN THE PRESENCE AND ABSENCE OF OXYGEN

BY EDGAR J. WITZEMANN

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Nef[1] divided the effects of alkali upon sugars into two groups, (1) the more profound reactions involving the destruction or oxidation of the sugars and (2) the less profound effects involving merely transformations, such as the isomerization phenomena described by Lobry de Bruyn and Alberda van Ekenstein.[2] Woodyatt[3] has pointed out the close parallelism between these two groups of effects and the effects exerted on sugars in the normal and diabetic organism, respectively. The diabetic accomplishes transformations of Type 2 very effectively, but fails to a greater or less extent in accomplishing effects of Type 1. When the diabetic is supplied with insulin his lost capacity for transformations of Type 1 can be restored. From this it might appear that insulin acts upon glucose *in vivo* as strong alkalies act upon it *in vitro*. There is, however, no reason for thinking that insulin is a strong alkali like potassium hydroxide.

Recent experiments on the behavior of ammonium hydroxide, in comparison with potassium and sodium hydroxides, on the oxidation of butyric acid with hydrogen peroxide,[4] give a basis for the belief that catalyses attributed to the hydroxyl-ion concentration of the alkali used are also influenced under suitable conditions by other factors such as the nature of the positive ion. It was in pursuit of the idea that somewhat similar relations might hold for the alkali effects of Type 1 that some preliminary fundamental experiments upon the action of guanidine were made.

The experiments here described were carried out before insulin became known and are offered in the present form because they may have considerable interest in other ways than those suggested above. The results in general were indecisive because the effects of guanidine on glucose proved to be much like those of potassium hydroxide.

[1] Nef, *Ann.*, **403**, 204 (1914). See also *Ann.*, **357**, 214 (1907); **376**, 1 (1910).

[2] de Bruyn and van Ekenstein, *Rec. trav. chim.*, **14**, 158, 203 (1895); **15**, 92 (1896); **16**, 257, 262 (1897).

[3] Woodyatt, *J. Biol. Chem.*, **20**, 129 (1914).

[4] Witzemann, *ibid.*, **49**, 123 (1921).

After the experiments were under way, the interesting study of Morrell and Bellars[5] on the guanidine hexosates was found. Their work relates mainly to transformations of Type 2, while the present experiments have been directed mainly to determine the extent and scope of reactions of Type 1 as brought about by guanidine.

Experimental Part

Optical and Reducing Properties of Guanidine-Glucose Solutions.—To 4.76 g. of free guanidine[6] in 160 cc. of water was added 2 g. of pure glucose. Half of this solution was placed in the incubator at 37°. The other half was kept at room temperature. The solution as prepared contained 1.62 g. of glucose as derived from its optical rotation and 1.95 g. of glucose as determined by the Benedict-Osterburg reduction method.[7]

Three days later the solution kept at room temperature showed a rotation of —0.08° in a 10cm. tube; 0.80 g. of sugar by the Benedict-Osterburg method, that is, 20.0% of the reducing power had disappeared, indicating that 20% of the sugar used had been destroyed. The solution kept in the incubator for three days showed a rotation of 0.00° in the same tube and 0.20 g. of sugar by the Benedict-Osterburg method, that is, an 80% loss in sugar content.

Both solutions became yellow on standing. That at 37° reached its maximum color after about 24 hours and was distinctly yellow. The one kept at room temperature required three or four days to become definitely yellow and reached a maximum in about a week.

Portions of these solutions were analyzed before and after hydrolysis with an excess of hydrochloric acid but no evidence of the formation of non-reducing polysaccharides was obtained.

Parallel experiments were carried out with a similar glucose solution containing 4.52 g. of potassium hydroxide and 2 g. of glucose in 160 cc. The results were very similar. The portion kept at room temperature for three days showed a polariscopic reading of —0.01° in the same tube and 0.712 g. of sugar by the Benedict-Osterburg method, that is, about 29% loss in sugar content as measured by reducing power.

The darkening of these solutions at 37° was about 5 to 6 times greater than with the same molecular concentration of guanidine at 37°. On the other hand, the yellowing with potassium hydroxide at room temperature was very much less than with guanidine. In a week the potassium hydroxide showed a scarcely perceptible effect, while with guanidine almost the maximum effect was developed in this time. The relations observed are qualitatively represented in the diagram and show that there is a distinct difference in the action of these two bases upon glucose as indicated by the qualitative differences in the formation of sugar tar.[8]

The experiments described above were repeated at concentrations of alkali corresponding to 0.1 *N* but the second series did not develop any additional facts. The

[5] Morrell and Bellars, *J. Chem. Soc.*, **91**, 1010 (1907).

[6] This was prepared according to the method used by Morrell and Bellars.

[7] Benedict and Osterburg, *J. Biol. Chem.*, **34**, 195 (1918).

[8] The specificity suggested in this diagram is especially interesting when considered in its biological relations. From the standpoint of living organisms the sugar catalyst that has the lowest temperature coefficient would be best suited for the conditions of life in general. The results represented in the diagram show that guanidine apparently fulfils this requirement much better than potassium hydroxide. Guanidine would be available for both warm- and cold-blooded animals, while a catalyst such as potassium hydroxide would perhaps be out of the question in one of them.

action of ammonium hydroxide and guanidine carbonate upon glucose was also observed at the same time.

With a solution containing 1.43 g. of guanidine carbonate and 1.00 g. of glucose in 160 cc. of water the rotation changed from $-0.31°$ to $-0.24°$ in a 10cm. tube and the reducing sugar by the Benedict-Osterburg method from 1.03 g. to 0.936 g. when kept at room temperature for five days. At 37° the values after five days were for the rotation $+0.02°$ in the same tube and for the reducing sugar content 0.666 g.

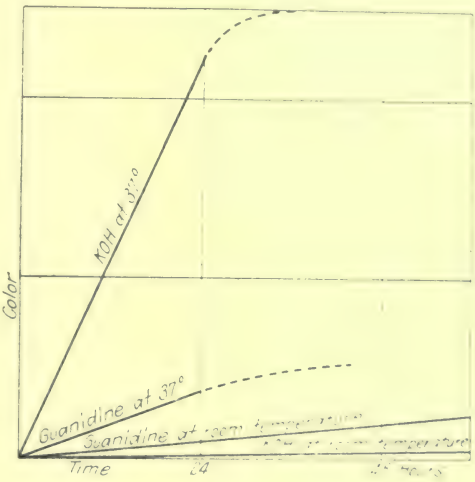

Fig. 1.

With 1 g. of glucose in 160 cc. of 0.1 *N* ammonium hydroxide the rotation remained unchanged at room temperature for five days while the sugar content dropped from 1.02 g. to 0.876 g. as determined by the Benedict-Osterburg method. A similar solution at 37° showed a rotation of $-0.21°$ to $-0.25°$ at the beginning and a reducing sugar content of 0.718 g. after five days. No yellowing occurred with either reagent.

The results show that even guanidine carbonate acts upon glucose giving rise to both mutarotation and sugar destruction.

The behavior of potassium hydroxide in the experiments described above is well known. Morrell and Bellars studied in detail the mutarotation phenomena caused by guanidine. The loss in reducing power of the solutions was measured by them in terms of the loss in alkalinity on the well-established assumption that salts of polyhydroxy acids are formed by rearrangements of the various isomeric sugars present in an alkaline solution of hexose. The difference in the coloring of glucose solutions with potassium hydroxide in comparison with free guanidine was the only definite indication of marked differences in the behavior of these two bases and could be established only by parallel observations.

The action of these bases will have to be studied with care and in detail by some method like that used by Nef[1] before anything definite can be said about the relative quantitative importance of the various types of chemical processes that occur in these guanidine and guanidine carbonate solutions.

Guanidine as an Oxidizing Catalyst

1. Using Hydrogen Peroxide.—It was a matter of considerable interest and importance to explore the capacity of guanidine to catalyze oxidation in order to determine to what extent its effects resemble those of sodium and potassium hydroxides under similar conditions.

The free base from 1.65 g. of guanidine carbonate in 25 cc. water was liberated with barium hydroxide solution, leaving a small amount of the carbonate in excess; 0.1 g. of glucose was added and the solution made up to 75 cc.; 25 cc. of this solution was mixed with 25 cc. of 2.1% hydrogen peroxide. After four days at room temperature the solution was found

to be free from peroxide. The solution was then oxidized completely with potassium permanganate by the method previously described,[9] and the permanganate consumed calculated to its glucose equivalent, namely, 0.281 g. of glucose in this case (0.33 g. used); 25 cc. of the original solution was analyzed in the same way and showed a permanganate consumption equivalent to 0.335 g. of glucose (0.33 g. used). Although peroxide was used in large excess only a small amount of oxidation took place. The evolution of oxygen during the oxidation was plainly visible. The guanidine + glucose solution was yellow in color while the yellow color was absent from the portion to which peroxide had been added. In this experiment the behavior of guanidine resembles that of sodium or potassium hydroxide.

The above experiments were repeated several times and about the same results were obtained. Sugar determinations by the Benedict-Osterburg method showed that at room temperature these solutions lose from 50 to 85% of their reducing power in four or five days. When kept at 37° they contained no reducing substance at all after five days. This result taken in conjunction with the results on the oxidation shows that guanidine, like potassium hydroxide, is a better catalyst for the destructive rearrangements of glucose than for oxidation.

2. *Using Air.*—Sixty cc. of solution containing 3.72 g. of guanidine and 1.0 g. of glucose in 125 cc. was subjected to aëration at room temperature by placing it in a gas wash-bottle and drawing an active stream of carbon dioxide-free air through it with a suction pump; 60 cc. was set aside for the control analysis.

A solution of 3.50 g. of potassium hydroxide and 1.0 g. of glucose in 125 cc. of water was treated in the same way and placed in the same suction train. Air was circulated for 48 hours in the course of 12 days. The total permanganate consumed by all four solutions was then determined. The permanganate consumed by the experiment using guanidine plus air was 13% less than in the blank; with the experiment using potassium hydroxide plus air it was 6% less than in the blank. Apparently guanidine catalyzes oxidation by air a little more than potassium hydroxide. Both guanidine solutions became yellow, while the potassium hydroxide solutions remained colorless.

3. *Using Air Plus Manganese Hydroxide.*—The preceding experiments were repeated with the addition of a solution containing 0.05 g. of manganese chloride to each experiment. On circulating air the pale yellow-to-orange solutions very quickly became a dark brown. After air had been passed through for two hours the solutions were allowed to stand over two days. Both mixtures that were being aerated then contained a flesh-pink precipitate, and the supernatant solution in the guanidine experiment

[9] Witzemann, *J. Biol. Chem.*, **45**, 1 (1920).

was almost colorless. The solution in the potassium hydroxide experiment was light brown. When air was again passed through the potassium hydroxide mixture, it darkened more quickly than the other. After two hours both had become equally dark red-brown. Decolorization was almost complete in 0.5 hour. The potassium hydroxide solution decolorized faster than the guanidine solution. These solutions after standing in the decolorized condition for some time showed a much darker color at the top of the solution where it was in contact with air. After the solution had been thus darkened several times a day for a week the decolorization became quite slow, indicating that the system was behaving differently. The solutions were then analyzed as before. The results were approximately the same as in Expt. 2. About 10% of the glucose was oxidized in both cases and guanidine seemed to function a little better than potassium hydroxide. The impression was obtained that the oxidation was considerably accelerated by the presence of the manganous hydroxide, considering the time during which air was circulated, but no satisfactory quantitative data were obtained. In any case the manganous hydroxide, besides functioning as an oxygen acceptor, served as an interesting indicator of the changes occurring in the solution. At first, compounds were present that took up oxygen rapidly, but the transformation of these compounds into others (presumably polyhydroxy acids), that are not readily oxidizable under these conditions, takes place much more rapidly than oxidation, and the solution then ceases to utilize appreciable amounts of oxygen. The same result was obtained with both potassium hydroxide and guanidine and is typical for such bases as sodium and potassium hydroxide.

Summary

The results described in this paper show that guanidine, which is an organic base that is comparable to sodium and potassium hydroxide, so far as the velocity of saponification of ethyl acetate[9] is concerned, is also comparable to these bases in its action upon glucose, in both the presence and the absence of oxygen or hydrogen peroxide. Guanidine causes a polymerization accompanied by yellowing more readily than potassium hydroxide, but this reaction was much less influenced by temperature than in the case of potassium hydroxide. The latter effect was the only difference in the behavior of these two bases that was large enough to be suspected of being specific.

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THE ACTION OF AMMONIUM HYDROXIDE AND OTHER ALKALINE COMPOUNDS UPON INSULIN.*

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(Received for publication, August 7, 1923.)

This paper is a report upon our observations on the lability[1] of insulin in the presence of alkali. These experiments were undertaken, first, in order to determine the influence of reaction conditions upon the activity of insulin, as a rational preparation for later attempts to isolate a definite chemical compound having insulin properties; and second, in order to obtain some hint, if possible, as to the chemical nature of the group or groups which contribute the particular property which characterizes insulin.

1. Action of Ammonium and Potassium Hydroxides on Insulin.—In Tables I and II are given the results of the action of ammonium hydroxide and potassium hydroxide upon insulin, which we prepared from fresh beef pancreas by the Collip method. The preparations were injected into rabbits that had been fasted during the 18 hours previous to injection. The blood sugar concentrations were determined before and again 2 hours after injection, unless the rabbits suffered convulsions, in which case

* We wish to express our gratitude to various executives in Swift and Co., Chicago, for supplying us, without cost, with the beef pancreas used in this and other work upon insulin done in the laboratory.

[1] Best and Macleod (Best, C. H., and Macleod, J. J. R., *J. Biol. Chem.*, 1923, lv, p. xxix) say that: "Moderate degrees of alkalinity (to litmus) do not affect the strength of insulin during a period of 6 minutes at room temperature. We cannot as yet state the degree of alkalinity which insulin can withstand." This is the only statement that we have found in the literature on the behavior of insulin with alkali. Since this paper was sent in for publication Dudley (Dudley, H. W., *Biochem. J.*, 1923, xvii, 676) has published some data on the destruction of insulin by NaOH solutions and by boiling Na_2CO_3 solutions.

TABLE I.

Experiments with Ammonium Hydroxide.

Injected 1 cc. into ±2 kilo fasted rabbits.

No.	Material.	Date.	Blood sugar.		Remarks.
			Before.	After.	
			per cent	*per cent*	
1	1.5 cc. insulin No. 45 + 2.5 cc. 1.0 N NH_4OH + 1.0 cc. H_2O. (Mixture 0.50 N NH_4OH.) Started Mar. 28.	Mar. 29	0.20	0.08	After 2 hrs.
		Apr. 3	0.14	0.14	" 2 " Acidified the solution with HCl Apr. 4.
		" 5	0.11	0.02	After 1 hr. (in convulsions).
2	Started above experiment again on Apr. 5.	" 7	0.12	0.05	" 2 hrs.
		" 12	0.12	0.07	" 2 " Acidified with HCl Apr. 12.
		" 13	0.11	−0.02	" 1½ " (in convulsions).
3	Same experiment started again Apr. 7.	" 9	0.14	0.08	" 2 "
		" 12	0.12	0.06	" 2 " Acidified with HCl Apr. 12.
		" 13	0.13	−0.02	" 45 min. (in convulsions).
4	1.5 cc. insulin No. 52 + 3.5 cc. 1.0 N NH_4OH. Started May 14.	May 16	0.12	0.13	" 2 hrs.
		" 17	0.14	0.12	" 2 " Acidified with HCl and injected 1 hr. later May 18.
		" 18	0.12	0.08	After 2 hrs.
		" 19	0.10	0.08	" 2 "
		" 21	0.10	0.05	" 2 "

5	Started again with No. 52 May 19.	May 21	0.10	0.03	After 2 hrs.
		" 22	0.11	0.03	" 2 " (in convulsions)
6	1.5 cc. insulin No. 47 + 3.5 cc. 1.0 n NH_4OH. Started May 19.	" 21	0.11	0.06	" 2 "
		" 22	0.16	0.12	" 2 "
		" 23	0.12	0.08	" 2 " Acidified with HCl and injected at once May 23.
		" 23	0.12	−0.02	After 2 hrs. (in severe convulsions).

blood was taken when convulsions were first observed as stated in the tables under the heading, "Remarks." On the dates indicated the alkaline solutions were acidified with hydrochloric acid (*i.e.* made distinctly acid to litmus) and subsequently injected again to determine if the insulin activity had been restored. That 0.3 cc. of the preparations used in these experiments always produced pronounced convulsions was determined by control experiments with the preparations in which 1.5 cc. were diluted with 3.5 cc. of water so that 1 cc. of dilute solution could be injected as in the alkali experiments. These control results are not given.

In Experiment 1 the insulin had become completely inactive in 6 days. When it was acidified, on the supposition that a reversible reaction is involved rather than a destructive reaction such as saponification or hydrolysis, the animal was thrown into convulsions a little sooner than it normally was by the unchanged original preparation. The blood sugar was also reduced to the level characteristic of convulsions. This experiment was the first one made and it was some time before we obtained the same result again.

In Experiment 2 the insulin activity was reduced considerably in a week. Since about 0.2 cc. of the original preparation lowered blood sugar to 0.03 per cent and produced convulsions, it was about 50 per cent inactivated in this experiment. When acidified it recovered its original strength fully. The same result was obtained in Experiment 3.

With insulin preparation No. 52 a little more ammonium hydroxide was added and the insulin became completely inactive in 2 days. This was confirmed on another rabbit the next day. The solution was then acidified and injected an hour later. Its activity was partially restored. It remained unchanged when injected the next day, but 2 days later had apparently regained somewhat more of its original activity.

In Experiment 5 we attempted to repeat No. 4, but the insulin lost nothing in activity in 3 days although the materials used were all portions of those that had been used in Experiment 4. Out of 30 or more experiments we obtained this result but once. It may have been due to the loss of ammonia around a loose stopper.

TABLE II.

Experiments with Potassium and Sodium Hydroxides.

Injected 1 cc. into ±2 kilo rabbits fasted 18 hours.

No.	Material.	Date.	Blood sugar.		Remarks.	
			Before.	After.		
			per cent	*per cent*		
7	1.5 cc. insulin No. 45 + 2.5 cc. 1.0 N KOH + 1.0 cc. H_2O. (Mixture 0.50 N KOH.) Started Mar. 28.	Mar. 29	0.15	0.12	After 2 hrs.	Acidified with HCl Apr. 4.
		Apr. 5	0.13	0.13	" 2 "	
8	Started Experiment 7 again Apr. 7.	" 9	0.13	0.12	" 2 "	Acidified with HCl Apr. 10.
		" 11	0.13	0.13	" 2 "	
9	1.5 cc. insulin No. 45 + 2.5 cc. 0.1 N KOH + 1.0 cc. H_2O. (Mixture 0.05 N KOH.) Started Apr. 14.	" 16	0.13	0.05	" 2 "	
		" 19	0.11	0.06	" 2 "	Acidified with HCl Apr. 19.
		" 20	0.10	0.06	" 2 "	
10	Experiment 9 with 0.1 N NaOH. Started Apr. 14.	" 16	0.11	0.05	" 2 "	
		" 19	0.14	0.05	" 2 "	
11	Repeated Experiment 10 with insulin No. 52. Started May 14.	May 16	0.11	0.06	" 2 "	
		" 17	0.14	0.12	" 2 "	Acidified with HCl May 18.
		" 19	0.10	0.10	" 2 "	

In Experiment 6 a much older preparation was practically entirely inactivated and restored to full strength when injected at once after acidifying. The last two injections in this experiment were made within a few minutes of each other.

The results of the experiments with potassium hydroxide require little comment. In Experiments 7 and 8 inactivation was complete and not reversible upon acidification. In Experiments 9 and 10, which contained one-tenth as much alkali, inactivation was slower but, as found in Nos. 9 and 11, irreversible upon acidification even after standing 2 days. In Experiment 11 inactivation was complete after 3 days, although in Experiment 10 inactivation was not well under way even in 5 days.

2. Action of Disodium Phosphate, Sodium Carbonate, and Bicarbonate on Insulin.—Some experiments were made on the action of these compounds upon insulin. The experiments were not extended since no pronounced effects were observed. In general, the prolonged action of these substances in the concentration used diminished the activity of the insulin enough to do away with the symptoms of convulsions although the blood sugar was reduced to about the level for convulsions. When acidified, however, these solutions that had been somewhat "dampened" by the alkali compounds were restored to full activity. In fact, they often seemed to be stronger in their effects than the controls with the untreated insulin.

3. The Velocity of Inactivation and Reactivation of Insulin at Room Temperature.—In order to determine the velocity of reactivation of insulin completely inactivated by ammonium hydroxide, the preparation in Experiment 6 was acidified and injected as quickly as possible afterward (*i.e.* within a minute or so). The results showed that insulin was restored to full activity by the time it had been absorbed from the point of injection. In the rabbit that received the inactive preparation there was a slight reduction of blood sugar, indicating that perhaps a little unchanged insulin remained in solution. In the other rabbit, this preparation, after having been acidified, behaved as though it had never been inactivated. A similar and more perfect result of this kind is recorded in Experiment 19. The results on the action of ammonium hydroxide show that insulin requires at least several days to be completely inactivated

TABLE III.

Experiments with Sodium Phosphate, Carbonate, and Bicarbonate.

Injected 1 cc. into ±2 kilo rabbits fasted 18 hours.

No.	Material.	Date.	Blood sugar.		Remarks.
			Before.	After.	
			per cent	*per cent*	
12	1.5 cc. insulin No. 45 + 3.5 cc. M/3 Na_2HPO_4. Started Mar. 28.	Mar. 29	0.11	0.02	After 1½ hrs. (in convulsions)
		Apr. 3	0.15	0.09	" 2 "
13	Repeated Experiment 12. Started Apr. 7.	" 12	0.14	0.07	" 2 " Acidified with HCl Apr. 12.
		" 13	0.14	−0.02	" 1 hr. (in convulsions).
14	1.0 cc. insulin No. 52 + 2.5 cc. 5 per cent Na_2CO_3 solution. Started May 10.	May 11	0.11	0.04	" 1½ hrs. (" ").
		" 15	0.09	0.03	" 1½ " (" ").
15	1.0 cc. insulin No. 52 + 1.0 cc. 5 per cent Na_2CO_3 solution. Started May 10. (Injected 0.6 cc.)	" 11	0.11	0.07	" 2 "
		" 15	0.10	0.03	" 1¼ " (in convulsions).
16	1.5 cc. insulin No. 45 + 3.5 cc. M/3 $NaHCO_3$ solution. Started Apr. 7.	Apr. 12	0.13	0.03	" 2 " Acidified with HCl Apr. 12.
		" 13	0.11	−0.02	" 1¼ " (in convulsions).

in our experiments, but does not require more than an hour or so, at most, to be completely reactivated.

4. Attempts to Catalyze the Action of Alkali on Insulin.—In Section 6 the action of ammonium hydroxide upon insulin is discussed as a keto-enol rearrangement. As is well known, such rearrangements are subject to catalytic influence in both directions. We were therefore interested in determining to what extent the inactivation of insulin by alkalies is subject to catalysis.

In a study of the action of alkali upon the oxidation of butyric acid[2] it was found that although potassium hydroxide alone is incapable of catalyzing the oxidation of butyric acid it promotes the action of ammonium hydroxide and may be substituted for part of it. Our first experiments were therefore made with mixtures of ammonium and potassium hydroxides.

The result in Experiment 18, especially, was surprising. The quantity of potassium hydroxide added (0.0028 gm.) was only one-fifth of that added in Experiment 10 in which insulin was not much inactivated even after some days. The effect of the addition of these small amounts of potassium hydroxide was to catalyze the permanent inactivation of insulin by ammonium hydroxide and to convert the reversible ammonium hydroxide effect into the irreversible potassium hydroxide effect. The result was a catalysis of the destructive reaction of alkali by ammonium hydroxide.

When Experiment 18 was repeated it was confirmed several times and then we finally obtained the results given in Experiment 19. This result is nearly 100 per cent different from that of the preceding experiment, and constitutes a catalysis of the reversible ammonium hydroxide effect.

Similar experiments were made using only 0.01 cc. of 1.0 N KOH, but the results so far obtained show no distinctive difference in comparison with the results obtained with ammonium hydroxide alone.

Other experiments were made substituting 0.2 to 1.75 cc. of the 1.0 N NH_4OH with 5 per cent Na_2CO_3. As near as we can tell the most frequent effect of the Na_2CO_3 is to prevent the inactivation of insulin by ammonium hydroxide.

[2] Witzemann, E. J., *J. Biol. Chem.*, 1921, xlix, 123.

TABLE IV.

Experiments with Mixed Alkalies.

Injected 1 cc. into ±2 kilo rabbits fasted 18 hours.

No.	Material.	Date.	Blood sugar.		Remarks.
			Before.	After.	
			per cent	*per cent*	
17	1.5 cc. insulin No. 47 + 3.32 cc. 1.0 N NH_4OH + 9.18 cc. 1.0 N KOH. Started May 24.	May 25	0.12	0.13	After 2 hrs. Acidified May 25.
		" 26	0.14	0.13	" 2 "
		" 28	0.14	0.14	" 2 "
18	1.5 cc. insulin No. 47 + 3.45 cc. 1.0 N NH_4OH + 0.05 cc. 1.0 N KOH. Started June 6.	June 7	0.13	0.14	" 2 " Acidified June 7.
		" 8	0.12	0.12	" 2 "
19	Repeated Experiment 18 with insulin No. 49. Started June 27.	" 28	0.13	0.12	" 2 " Acidified just before injection.
		" 29	0.13	0.04	" 2 " (no convulsions).

In brief, these preliminary results indicate that the effects of mixed alkalies do give rise to catalytic effects subject to the same unexplained vagaries as the simple alkalies.

5. The Nature of the Chemical Action of Alkaline Compounds on Insulin.—The results in the preceding sections show two distinct types of effects of alkaline compounds upon insulin, and possibly a third.

a. One of these is the striking reversible inactivation of insulin by ammonium hydroxide, which will be briefly discussed in the next section.

b. Another reaction which is typically obtained with potassium and sodium hydroxides is an irreversible destructive reaction. The writers are under the impression that this reaction is possibly an autohydrolysis due to the alkali, analogous to the autohydrolysis of alkali caseinates studied by Walters,[3] or some other reaction not reversible by acid.

c. The peculiar effect of sodium carbonates and phosphate was that they did not often (in the 25 to 30 observations made) appear to modify greatly the capacity of insulin preparations to lower the blood sugar, but did generally diminish what may perhaps be considered to be secondary effects; namely, convulsions. On the contrary, when the same preparations were acidified they appeared to cause somewhat greater lowerings in the blood sugar and more profound convulsions than the same preparation gave in the control experiments. Acidification of the untreated insulin preparation used in the control experiments did not give any pronounced increase in the effect. We are disposed to consider that in these alkaline preparations the chemical group in the insulin molecule that gives rise to these secondary effects becomes masked by condensing with other molecules present in the preparation, but that this does not effectively interfere with the action of the same or another group upon the sugar of the blood.

Our experiments with ammonium hydroxide as a whole gave three types of results: (1) the insulin was not appreciably inactivated; (2) the insulin was reversibly inactivated; and (3) the insulin was irreversibly inactivated. The first type of results

[3] Walters, E. H., *J. Biol. Chem.*, 1912, xii, 43; *cf.* also Mann, G., Chemistry of the proteids, London, 1906, 90, 337.

was obtained occasionally and is mentioned here for completeness because we are not yet sure that we can always avoid it and get the second type of effect. More than half the time we got the mixed effect of Types 2 and 3. Our difficulty was to get to Type 2 without going on to Type 3. We were, therefore, unable to predict in any given case which result would be obtained, even when the same materials were used. Owing to the impossibility of controlling all the variables that seem to be involved (such as the purity of the insulin, especially) we have contented ourselves with a qualitative survey of this aspect of the problem for the present, and hope to get a clearer insight into the factors involved by widening rather then intensifying our attack. Enough of our characteristic results have been included in this paper to illustrate all the effects observed.

6. Possible Interpretation of the Action of Ammonium Hydroxide upon Insulin.—The available chemical results upon insulin are as yet far too meager to provide a satisfactory basis for the interpretation of the reversible transformation of insulin described above. It should, however, not be misleading to consider briefly what the results obtained suggest concerning the constitution of insulin.

The definite chemical facts developed in this paper clearly suggest that the reversible effect of ammonium hydroxide involves a tautomeric rearrangement such as $\cdot CO \cdot NH \cdot \rightleftarrows C(OH):N \cdot$ or $\cdot CO \cdot CHR(\text{or H}) \rightleftarrows \cdot C(OH):CR(\text{or H})$. These two groups are mentioned solely because the first is the most abundantly well known tautomeric group occurring in protein-like compounds, such as insulin appears to be,[4] and the second is the hitherto most abundantly studied rearrangement of this kind.[5]

When the results are considered in this way, interesting and important analogies between insulin and other biochemical catalysts are suggested. Thus the pioneering results of von Euler and Svanberg[6] on the reactivation of "poisoned" or inactivated

[4] Witzemann, E. J., and Livshis, L., *J. Biol. Chem.*, 1923, lvii, 425.

[5] Meyer, V., and Jacobson, P., Lehrbuch der organischem Chemie, Leipsic, 2nd edition, 1902, iii, 1137. Meyer, K. H., *Ann. Chem.*, 1911, ccclxxx, 212; Meyer, K. H., and Kappelmeier, P., *Ber. chem. Ges.*, 1911, xliv, 2718; 1912, xlv, 2843. Knorr, L., Rothe, O., and Averbeck, H., *Ber. chem. Ges.*, 1911, xliv, 1138. Dimroth, O., *Ann. Chem.*, 1904, cccxxxv, 14.

[6] von Euler, H., and Svanberg, O., *Fermentforsch.*, 1919–20, iii, 330.

enzymes are of special interest in this connection. The poisoning effect of heavy metals ($HgCl_2$ and $AgNO_3$) upon invertase is completely reversible. Their results on the reversible inactivation of invertase by eleven amines[7] led them to consider that the aldehyde group determines this property of invertase. Meanwhile, Wöker and Maggi[8] consider for entirely different reasons that the effects of peroxidase, catalase, reductase, and diastase are definitely due to the aldehyde group and have proposed formaldehyde as a chemical model for these four types of enzymes, since it shows all these types of chemical activity.

The results cited above as well as other work that is not mentioned are interesting in connection with insulin mainly because they suggest ways of extending the study of the labile group involved in the reversible transformation described.

SUMMARY.

The results described in this paper show that insulin is more or less completely inactivated at room temperature by 0.5 or 0.7 N ammonium hydroxide in the course of some days and that its original activity is usually quickly restored on acidifying the ammoniacal solution with hydrochloric acid. It was also found that sodium and potassium hydroxides even at 0.1 N concentration inactivate insulin irreversibly at room temperature. Sodium carbonate and bicarbonate and disodium phosphate had almost no action upon insulin. Some exploratory experiments on the effects of mixed alkalies upon insulin indicate that the above reactions are also subject to secondary accelerative and retarding effects depending on the mixture used.

The known facts concerning the reversible inactivation of insulin by ammonium hydroxide suggest a tautomeric rearrangement. Nothing definite can yet be said as to the nature of the groups that would be involved in this rearrangement.

[7] von Euler, H., and Svanberg, O., *Formentforsch.*, 1920–21, iv, 29, 54, 143.

[8] Wöker, G., and Maggi, H., *Ber. chem. Ges.*, 1919, lii, 1594. Maggi, H., *Formentforsch.*, 1919, ii, 304.

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THE ACTION OF PROTEOLYTIC ENZYMES UPON INSULIN.

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(Received for publication, June 29, 1923.)

Insulin is the name applied to a solution obtained from the pancreas of animals which has the power of increasing the combustion of carbohydrates in diabetes. Just what this solution contains that has this property is by no means entirely known. In this paper results obtained by the action of commercial proteolytic enzymes on insulin are described. The results obtained indicate that insulin is protein-like in nature.

Protein Nature of Insulin.

Insulin preparations obtained by the Collip method show the typical qualitative reactions of proteins. This means, if insulin itself is not a protein or polypeptide, that it is contaminated with alcohol-soluble protein. The simplest and most obvious way to determine whether the substance, having the properties of insulin, is thus contaminated with protein is to subject the preparation to the action of proteolytic enzymes[1] by which the presumably contaminating proteins would be removed by hydrolysis. If the solutions still showed activity it would prove that the protein was merely an impurity.

A series of experiments were done with trypsin, papain, and pepsin, using Lilly and Co.'s iletin and our own insulin (prepared by Collip's method[2]).

[1] Banting and Best (Banting, F. G., and Best, C. H., *J. Lab. and Clin. Med.*, 1921-22, vii, 251) state that pancreatic juice destroys the activity of the liquid pressed out of macerated degenerated dog pancreas. This is the only statement concerning the behavior of insulin preparations toward proteolytic enzymes that we have been able to find in the literature.

[2] Until about Mar. 15, 1923, our own insulin was all required for clinical use, but we were sometimes given certain Lilly and Co. preparations for chemical experimentation. Since that date we have received no such preparations and have used our own.

Action of Trypsin on Insulin.—7.5 cc. of a Lilly and Co. preparation of iletin (No. 67569.725708) containing 40 units in 5 cc. (label) were diluted with an equal volume of water. Of this 5 cc. were placed in a flask as Experiment 1. The remaining distinctly acid solution was neutralized to litmus with $NaHCO_3$ and a small excess added. This gave a solution having the optimum reaction for peptone hydrolysis with trypsin; *i.e.*, the reaction of a $NaHCO_3$ solution containing free CO_2. Half of this solution constituted Experiment 2. To the remainder 0.01 gm. of trypsin (powder) was added. After standing 42 hours at room temperature 1 cc. of these solutions was injected into three rabbits (approximately 2 kg.) that had been fasted for 18 hours.

First Series.

No.	Material used.	Blood sugar.		Blood taken.
		Before.	After.	
		per cent	*per cent*	
1	5 cc. diluted iletin.....	0.13	0.043	After 1.5 hrs. (in convulsions).
2	5 " " " + $NaHCO_3$...	0.15	0.057	After 2 hrs. (no convulsions).
3	5 " " " + " + 0.01 gm. trypsin.....	0.14	0.13	After 2 hrs. (no convulsions).

Second Series.—The above experiments were repeated with our own preparation of insulin. 0.03 gm. of trypsin was suspended in 7.5 cc. of water. 6.0 cc. of insulin (No. 45, of which 0.3 cc. produced pronounced convulsions in less than 2 hours) were diluted with 4 cc. of water and treated with a slight excess of $NaHCO_3$.

The experiments were set up as stated in the table and after 42 hours 1 cc. portions of the solutions were injected into ±2 kg. fasted rabbits as before.

Second Series.

No.	Material used.	Blood sugar.		Blood taken.
		Before.	After.	
		per cent	*per cent*	
1	1.5 cc. insulin No. 45 + 3.5 cc. water.....	0.13	0.03	After 1.25 hrs. (in convulsions).

Second Series—Continued.

No.	Material used.	Blood sugar. Before.	Blood sugar. After	Blood taken.
2	2.5 cc. insulin + $NaHCO_3$ + 2.5 cc. water	0.12	0.023	After 1.25 hrs. (in convulsions).
3	2.5 cc. insulin + $NaHCO_3$ + 2.5 cc. trypsin solution	0.12	0.13	After 2 hrs. (no convulsions).
4	2.5 cc. insulin + $NaHCO_3$ + 2.5 cc. trypsin solution (boiled 5 min. before adding to destroy the enzyme)	0.14	0.037	After 2 hrs. (no convulsions).
5	2.5 cc. trypsin solution + 2.5 cc. water	0.15	0.14	After 2 hrs. (no convulsions).

Third Series.—The experiments in the second series were repeated using our own insulin (No. 52) of which 0.2 cc. produced convulsions in less than 2 hours.

Third Series.

No.	Material used.	Blood sugar. Before.	Blood sugar. After.	Blood taken.
		per cent	*per cent*	
1	1.5 cc. insulin No. 52 + 3.5 cc. water	0.10	0.03	After 1.25 hrs. (in convulsions).
	2.5 cc. insulin + $NaHCO_3$ + 2.5 cc. water	0.10	0.03	After 45 min. (died in convulsions).
3	2.5 cc. insulin + $NaHCO_3$ + 2.5 cc. trypsin solution	0.10	0.10	After 2 hrs. (no convulsions).
4	2.5 cc. insulin + $NaHCO_3$ + 2.5 cc. trypsin solution (heated 12 min. in boiling water to destroy the enzyme)	0.10	0.03	After 2 hrs. (no convulsions).
5	2.5 cc. trypsin solution + 2.5 cc. water	0.10	0.11	After 2 hrs. (no convulsions).

Action of Papain on Ilotin.—Similar experiments were made using the same Lilly and Co. preparation as before and commercial papain as the proteolytic enzyme. 7.5 cc. of ilotin were diluted

with 7.5 cc. of water and treated with a slight excess of $NaHCO_3$ since Oppenheimer[3] states that papain works best in nearly neutral solution.

After standing 45 hours at room temperature 1 cc. portions of the solutions were injected into ±2 kg. fasted rabbits as usual.

Papain Experiments.

No.	Material used.	Blood sugar.		Blood taken.
		Before.	After.	
		per cent	*per cent*	
1	5 cc. diluted iletin	0.11	0.025	After 1.5 hrs. (in convulsions).
2	5 " " " + $NaHCO_3$	0.12	0.030	After 1.25 hrs. (in convulsions).
3	5 " " " + " + 0.01 gm. papain	0.125	0.125	After 2 hrs. (no convulsions).
4	5 cc. water + 0.01 gm. papain	0.135	0.135	After 2 hrs. (no convulsions).

Action of Pepsin on Iletin.—The same Lilly and Co. preparation was tested with commercial pepsin. 7.5 cc. of iletin were neutralized with a slight excess of $NaHCO_3$. 0.3 gm. of pepsin were suspended in 0.2 per cent hydrochloric acid solution.

After 45 hours 1 cc. portions of the solutions were injected into ±2 kg. fasted rabbits as before.

Pepsin Experiments.

No.	Material used.	Blood sugar.		Blood taken.
		Before.	After.	
		per cent	*per cent*	
1	2.5 cc. iletin + 2.4 cc. water	0.11	0.025	After 1.5 hrs. (in convulsions).
2	2.5 cc. iletin + $NaHCO_3$ + 2.5 cc. water	0.14	0.024	After 1 hr. (in convulsions).
3	2.5 cc. iletin + $NaHCO_3$ + 2.5 cc. pepsin solution	0.13	0.10	After 2 hrs. (no convulsions).
4	2.5 cc. iletin + $NaHCO_3$ + 2.5 cc. pepsin solution (boiled 5 min. to destroy enzyme)	0.13	0.03	After 2 hrs. (no convulsions).
5	2.5 cc. pepsin solution + 2.5 cc. water	0.13	0.15	After 2 hrs. (no convulsions).

[3] Oppenheimer, C., Die Fermente und ihre Wirkungen, Leipsic, 4th edition, 1913, 604.

Interpretative Experiments.—The above experiments indicate that insulin is protein-like in nature. Before definitely concluding that this is true it was desired to criticize this result experimentally as completely as possible.

It was found in other experiments that 0.5 N NH_4OH inactivates insulin after 6 days at room temperature and that its activity is restored at once after slightly acidifying the solution with hydrochloric acid.[4] The recent work of von Euler and Myrbäck[5] and von Euler and Svanberg[6] showed that saccharase is reversibly "poisoned" by *p*-phenylenediamine, *p*-toluidine, aniline, and phenylhydrazine. Moreover, the activities of certain proteolytic enzymes are decreased by the presence of products of digestion.[7] It was therefore thought possible that insulin was inactivated in the above experiments by the digestion products of foreign protein present in its solutions.

Influence of Digestion Products on Insulin.—1.5 cc. of insulin (No. 45) + 3.5 cc. of water were treated with 0.01 gm. of trypsin at room temperature for 2 days. The flask was then immersed in a boiling water bath for 10 minutes. After cooling 1.5 cc. of insulin (No. 45) were added. The next day 1.3 cc. of the mixture were injected into a fasted 2 kg. rabbit. The rabbit developed convulsions in 2 hours. The blood sugar was 0.13 per cent before injection and 0.04 per cent 2 hours later. The dose as given was equivalent to 0.3 cc. of the undiluted insulin (No. 45) which always produced convulsions in about 2 hours and reduced the blood sugar to 0.02 to 0.04 per cent in 2 hours. This experiment shows that insulin is neither neutralized nor inactivated by the chemical action of the products of tryptic digestion of the proteins present in insulin prepared from beef pancreas. These results do not give any basis for modifying the conclusion that insulin is protein-like in nature.

Formol Titration of a Trypsin Digestion of Insulin.—If it could be shown that the "digestion" of insulin does not increase the formol titration of the solution it would indicate that the influence

[4] These observations will be described in full in a paper now in preparation.

[5] von Euler, H., and Myrbäck, K., *Z. physiol. Chem.*, 1923, cxxv, 297.

[6] von Euler, H., and Svanberg, O., *Arkiv mat. [illegible]*, 1920, 21, A, no. 29, 54.

[7] Oppenheimer,[3] p. 78.

of proteolytic enzymes was some chemical effect other than digestion.

The experiments were set up as follows:

No.	Material used.
1	7 cc. trypsin solution (0.025 gm. of trypsin) + 3 cc. water.
2	7 " " " (0.025 " " ") + 3 " insulin No. 45.
3	3 cc. insulin No. 45 + 7 cc. water.

After standing 2 days at room temperature these solutions were titrated by the usual formol titration method. When the results for Nos. 1 and 3 were added together and compared with those for digested insulin in No. 2 it was evident that the amino-acid content of No. 2 was increased corresponding to 1.73 cc. of 0.1 N NaOH; *i.e.*, about 50 per cent.

This shows that the digestion of insulin solution gives rise to titrable amino-acids, but of course this experiment alone does not indicate whether they were derived from protein impurities or from insulin.

Does the Protein Serve as a Colloid Protector?—It has been suggested that the protein in enzyme preparations acts as a colloid protector. This idea rests on such observations as those of Paal, who found that colloidal palladium suspended in proteins is rendered inactive as a catalyst for hydrogenation, when the protein is digested with enzymes. This did not occur when the protein was replaced by protalbic and lysalbic acids, which are not much digested by proteolytic enzymes. Waentig and Steche,[8] who destroyed catalase with trypsin, considered at first that possibly the protein colloid protector only had been digested. On the basis of later and exhaustive studies on catalase Waentig and Gierisch[9] concluded that this reservation does not apply, that catalase is really a protein and that its inactivation by proteolytic enzymes is not due to the destruction of a protective colloid.

Therefore, this suggestion can certainly not stand against the protein-like nature of insulin until some tangible basis for its support is discovered.

[8] Waentig, P., and Steche, O., *Z. physiol. Chem.*, 1913, lxxxiii, 315.
[9] Waentig, P., and Gierisch, W., *Fermentforsch.*, 1914–16, i, 165.

Discussion of Results Obtained with Proteolytic Enzymes.—The above results with trypsin, papain, and pepsin prove that these enzymes destroy the activity of insulin preparations. That this is not a simple neutralization is indicated by the fact that boiled trypsin and pepsin do not change the activity of insulin even on standing in contact with it for 42 hours and by the fact that digested insulin does not inactivate undigested insulin. The simplest conclusion from these results is that insulin itself is protein-like in nature.

Such a conclusion is in harmony with the existing knowledge concerning the best known enzymes, and there is no *a priori* theoretical reason for doubting its truth. In fact, since enzymes must be elaborated and manipulated within protoplasmic systems, it seems simple and logical to assume that they will have solubility relations well suited to this medium. All in all, similarity in constitution is one of the safest guarantees of solubility now known. We could, therefore, reasonably expect compounds, so indispensably related to protoplasmic activity as enzymes are, to have definite structural relationships to the proteins in their constitution. Such an arrangement would help to simplify the problem of organizing vital processes as it exists in higher organisms. Thus the unspecialized proteins of the cell would constitute the raw material from which enzymes could be elaborated at once when needed.

On the other hand, the idea that enzymes may generally be protein-like does not mean that they are not strikingly different in some respects from the rank and file of proteins.

The conclusion that insulin is protein-like in nature is contradicted by the results of Best and Macleod,[10] who isolated from fish (skate) an insulin preparation that does not give the color reactions for protein. They also state the following: "That the trace of protein in ox insulin preparations is an impurity is evidenced by the fact that after precipitation with phosphotungstic acid and removal of the latter by ether and barium the preparation retains its powers to cause hypoglycemia." On the other hand, all their preparations from ox pancreas gave the color reactions for proteins. The solid insulin preparation described by Doisy,

[10] Best, C. H., and Macleod, J. J. R., *J. Biol. Chem.*, 1923, lv, p. xxix.

Somogyi, and Shaffer[11] contained 14 per cent nitrogen and showed a high activity. If this material which separates at the isoelectric point is all insulin it is undoubtedly a protein.

Three Types of Insulin Preparations.

On the basis of our present knowledge there are at least three distinct types of insulin preparations.

1. The Protein Type.—This is the preparation of Doisy, Somogyi, and Shaffer.[11] We have not prepared insulin by their method, but we have regularly obtained a protein precipitate from our strong final aqueous solution. After precipitating the insulin with alcohol the extract was taken up in water and passed through a Berkefeld filter. On standing in the refrigerator a fine white "insulin" powder separated during the first few days. One such precipitate, after being washed with 80 cc. of distilled water in two portions, was suspended in 10 cc. of distilled water. Of this suspension 1 cc. was injected into a 2 kg. fasted rabbit. The blood sugar before injection was 0.113 per cent and 0.02 per cent 2 hours after injection; the rabbit was found in convulsions after 2 hours. This precipitate was afterwards dried out in a desiccator over $CaCl_2$ and was found to weigh 0.03 gm. This was again suspended in 10 cc. of distilled water after a month in the desiccator. On injecting 1 cc. of the suspension into a fasted rabbit convulsions appeared in 2 hours and the blood sugar dropped from 0.10 to 0.03 per cent. When the supernatant water, from which this active precipitate had been separated, was injected it produced no change in the blood sugar.

If insulin is adsorbed in this extremely fine protein powder it must be held with great avidity not to be removed by such prolonged action of so much water during the centrifuging of the fine precipitate.

The protein type of insulin differed from the two succeeding types in being insoluble in water and gave the blue-violet biuret test.

2. The Peptone-Polypeptide Type.—This preparation differs from the preceding in being easily soluble in water and in giving the red biuret test.

[11] Doisy, E. A., Somogyi, M., and Shaffer, P. A., *J. Biol. Chem.*, 1923, lv, p. xxxi.

The major portion of our insulin preparation does not separate as a solid. Our own insulin preparations, from which the above protein precipitate no longer separated after standing in the refrigerator for weeks, gave a violet-red biuret test, in which it was sometimes quite difficult to detect any violet tint at all. On the other hand, all Lilly and Co. preparations that we have tested gave an unmistakably violet colored biuret test. The colors were compared with the biuret test obtained with peptone (Chassaing, Paris), which gives a reddish violet tint and with peptone (Armour) which gives a bluish violet tint. Lilly and Co.'s iletin preparations gave colors resembling that of the peptone (Armour).

From the above tests we have concluded that the soluble insulin preparations that we have seen belong to this peptone-polypeptide type.

3. The Abiuret Type.—This type of insulin may be present as one of the components in the preceding type, but it deserves separate consideration because of the relative simplicity of its constitution. As quoted above, Best and Macleod obtained insulin from skate and from beef pancreas (by a special treatment) that gives neither the biuret test nor the qualitative reactions of protein. We have not investigated this type of insulin. It is obvious, however, that the synthesis of insulin will most likely be accomplished first with this type.

We do not wish to conclude too much from these tests, but there is certainly diversity enough in the behavior of the above preparations in the biuret test and in the solubility in water, even in those observed by us, to permit of concluding that insulin is a protein, a peptone, or a polypeptide,[12] depending upon which preparation was tested.

This diversity of characteristics and properties of insulin may seem surprizing at first, but is readily accounted for when we consider that insulin constitutes the "spoils" obtained by a certain process of "sifting" the "debris" of pancreas tissue broken down mechanically as much as possible. The "size" of the pieces obtained will depend on all the conditions involved in the material used and on the nature of the "sieve" or process of preparation used.

[12] Robertson (Robertson, T. B., The physical chemistry of the proteins, New York, London, Bombay, Calcutta, and Madras, 1918, 14) states that many polypeptides including tripeptides give typical biuret reactions.

If it is granted that the action of the proteolytic enzymes on insulin in the experiments described above was a true proteolytic hydrolysis, involving the breaking up of at least one amino-acid coupling and, therefore, presumably containing at least one amino-acid in its molecule, then the known facts will admit of two simple interpretations.

1. Insulin is a simple polypeptide that does not give a biuret reaction nor any of the typical protein reactions. It is, however, readily adsorbed by more complex protein derivatives and is brought down by them as in the "solid insulin" of Doisy, Somogyi, and Shaffer, and in various processes of precipitating insulin.

2. Insulin is a group or radical present in certain proteins which is easily hydrolyzed by proteolytic enzymes. This radical may occur in protein compounds of greater or less complexity and, therefore, its qualitative reactions will range all the way from a negative biuret, for the simplest group that still retains the characteristic properties of insulin, to those of a typical protein.

The second of the above interpretations includes the first in a different form. The first interpretation will be more attractive to those interested in eventually manufacturing insulin synthetically and in developing clearer views of the mechanics of its action, in as far as its effects can be ascribed to a given chemical constitution. The second interpretation conforms better with most of the things that we know about life and chemistry and is more likely to be correct because it is more general in its outlines. For instance, it permits us to consider that insulin is a chemical effect, that probably all living organisms have an insulin function, that the relative ease of isolation of the active grouping will vary from one organism to the other, that any undesirable secondary clinical effects may be controlled by preparations from other sources, that there is more than one chemical configuration possessing the insulin effect, and other interesting propositions.

If insulin is considered to be a specific chemical substance, the conclusion that it may be a polypeptide, peptone, or protein, depending upon the process used and the portion of the product tested, is unsatisfactory. If, however, the insulin effect is conceived of as associated with a certain group or grouping, the above interpretation indicates that this group persists in an effective form in several stages of protein breakdown. If this is true, it

may be found, when the facts are known, that the insulin effect will parallel the lipase effect, which is found under suitable conditions even with certain amino-acids as well as polypeptides and proteins,[13] but is, of course, best developed in lipase preparations. These experiments indicate that the parallelism cannot go down so far as the free amino-acids since the digestion products of insulin were found to have no insulin activity.

SUMMARY.

The fact that insulin preparations are completely inactivated by proteolytic enzymes (trypsin, papain, pepsin) indicates that the compounds present in the preparation, that give the qualitative reactions for proteins, are not all impurities, and that insulin itself is protein-like in nature. Attempts to break down this conclusion by other interpretative experiments have so far failed.

In discussing the available information upon the nature of insulin it is suggested that insulin is the "effect" of a certain group or grouping. This "effect" appears to be associated with a protein on the one hand and peptones and polypetides on the other.

If this interpretation of the known facts concerning insulin is correct, it does away with the contradictions concerning its nature, existing in the literature, and also conforms with the new facts presented in this paper.

[13] Falk, K. G., The chemistry of enzyme action, New York, 1921, 84.

Time Required for Disappearance of Intradermally Injected Salt Solution

Preliminary Report of Observations, with Special Reference to Cases of Edema

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TIME REQUIRED FOR DISAPPEARANCE OF INTRADERMALLY INJECTED SALT SOLUTION

PRELIMINARY REPORT OF OBSERVATIONS, WITH SPECIAL REFERENCE TO CASES OF EDEMA*

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If 0.2 c.c. of an 0.8 per cent. aqueous solution of sodium chlorid is injected intracutaneously in a normal child, a circumscribed snow-white elevation, in which the pores are accentuated, is immediately produced at the point of injection. Although the extreme whiteness disappears in from one half to one minute, the elevation persists for a considerable time. In some instances there is an area of erythema about the raised area which usually disappears in a few minutes.

It occurred to us that a study of the time required for the disappearance of the elevation, which we assume to be largely the injected salt solution, might give an indication of the tissue's avidity for water. We tested this out in a number of normal and diseased persons, most experiments being carried out under the observation of two.

TECHNIC

Two-tenths cubic centimeter of an 0.8 per cent aqueous solution of sodium chlorid is injected intracutaneously under aseptic precautions. Duplicate injections are made about 2 cm. apart either in the forearm or in the leg, or in both. In the forearm the flexor surface is used, and in the leg, either the inner surface of the calf or the lateral side of the anterior surface of the leg. The needle is inserted sufficiently superficially so that the lumen is visible through the skin. The end point

* From the Otho S. A. Sprague Memorial Institute Laboratory of the Children's Memorial Hospital.

of the disappearance of the elevation can best be determined by palpation unassisted by inspection, since color changes seen about the point of injection may cause confusion. It is necessary to disregard the very small traumatic elevation that is occasionally produced just where the needle enters the skin. This may persist after the large elevation, due to the injected salt solution, has disappeared.

Salt solution is injected rather than water for two reasons: (1) It is isotonic, and (2) the injection of distilled water is extremely painful.

In carrying out the experiments, it is noted that the duplicate elevations in the same part disappear prac-

TABLE 1.—*Control Cases*

Case	Age		Diagnosis	Average Disappearance Time in Minutes *		Pitting
	Yrs.	Mos.		Arm	Leg	
1	1	2	Fracture	56±	62±	None
2	2	...	Postoperative appendicitis	66+	65+	None
3	4	...	Preoperative knock-knee	69±	....	None
4	4	6	Preoperative circumcision	58	58±	None
5	6	9	Fracture	50	83±	None
6	8	...	Fracture	74+	72+	None
7	8	8	Fracture	74	94±	None
8	9	6	Fracture	68	103±	None
9	13	...	Head injury	70±	68+	None
10	Adult		Normal	80±	....	None
11	Adult		Normal	60+	....	None
12	Adult		Normal	70	....	None

* The plus sign indicates still palpable; plus-minus, still slightly palpable.

tically simultaneously. Any marked deviation from this is readily accounted for by a difference in the depth of the two injections. Practice has enabled us almost completely to eliminate this difficulty. Although the duplicate elevations in the same part disappear almost simultaneously, frequently there is a difference in the disappearance time in the arm as compared with the leg in the same individual.

The group of cases in Table 1 is given as a basis for comparison with results found in the pathologic cases. These patients were apparently normal except for the condition noted under "diagnosis." Several normal adult cases are also included. It is seen that the raised area produced by the injected salt solution persisted at least fifty minutes in every case.

PATHOLOGIC CASES

Various pathologic conditions showed decided reduction of the disappearance time, as compared with control cases. This is especially true in children who have a demonstrable edema. In these cases the disappearance of the elevation is at times very rapid. Table 2 records examples of cases of edema.

Table 2 shows that the disappearance of the raised area produced by injection of the salt solution is decidedly more rapid in cases of edema than in the controls of Table 1. The disappearance time is roughly in inverse ratio to the degree of the edema at the area

TABLE 2.—*Cases of Edema*

			Arm			Leg			
				Average Disappearance Time			Average Disappearance Time		
Case	Age in Yrs.	Diagnosis	Pitting	Min.	Sec.	Pitting	Min.	Sec.	Urine
13	10	Nephritis	Slight	14	50	Slight	7	45	Albumin + + + ; casts; R. B. C
14	8	Nephritis; endocarditis	None	38	..	Slight	10	..	Albumin +
15	10	Decompensated heart	None	1	8	Marked	..	35	Albumin + + + ; occas. R. B. C.; no casts
16	6	Endocarditis; anemia	None	30	..	Slight	17	..	
17	2½	Anemia; leukemia	Very marked	..	20*	Extreme	..	3*	Albumin, trace
18	2½	Pyonephrosis; anemia	None	30	..	Slight	15	..	W. B. C. + + ; albumin, trace

* Approximate; the disappearance was too rapid to time accurately

of injection. Attention is especially directed to the fact that in regions in which there is no edema that can be demonstrated by pitting, the disappearance is also more rapid than in the controls.

Table 3 records the results of tests made in Case 13 at intervals during a considerable period.

It is seen in Table 3 that, in a general way, the disappearance time decreases as the edema and the pathologic findings in the urine increase. At no time, even in the absence of pitting, did it come close to the time for the control group in Table 1. The gain and loss of body weight, in conjunction with the degree of the pitting, are given as an evidence of increased and decreased water retention. From March 28 to April 13, the increase of weight was evidently due to normal tissue

increase, since at this period the patient was doing better, and the edema, as determined by pitting, was decreasing.

COMMENTS

It is not our purpose in the present paper to discuss the physical or chemical factors which account for the difference in time required for disappearance of the elevations produced by the injected salt solution. We wish to call attention principally to the marked increase in rapidity of disappearance in cases of demonstrable

TABLE 3.—*Observations in Case 13*

	Arm			Leg				
		Average Disappearance Time			Average Disappearance Time			
Date, 1923	Pitting	Min.	Sec.	Pitting	Min.	Sec.	Urine	Weight of Patient in Pounds
2/21	None	5	55	Marked	3	5	R. B. C. ++++, Alb. ++, W. B. C. +, casts ++	..
2/22	....	..	..	Less marked	5	..	Alb. +++, casts ++, R. B. C. ++	..
2/23	None	8	..	Less marked	11	5	Alb. +++, casts ++, R. B. C. ++	2/24, 61
2/26	....	..	..	Marked	8	35	Alb. +++, casts ++, R. B. C. ++	..
2/27	None	4	30	Marked	7	..	.....	62
2/28	....	..	..	Marked	6	10	.....	63
3/ 2	None	8	20	Marked	3	55	Red	62½
3/ 6	....	..	..	Less marked	7	35	Red.; Alb., 14 gm. per liter	60
3/13	....	..	..	Definite	7	50	Much less blood	56
3/19	....	..	..	Slight	15	15	Alb., 0.75 gm. per liter 3/16	50¼
3/23	....	..	..	Slight	10	35	Albumin, trace	50
3/28	....	..	..	None	27	15	Albumin +	48
4/ 6*	....	..	..	Slight	26	45	Albumin +	50¼
4/13	....	..	..	None	24	45	Albumin, trace	50
4/24	Slight	14	50	Slight	7	45	Alb. +++, casts, R. B. C.	..
5/16	Slight	28	10	Slight	13	40	Albumin ++	58¼
5/24	None	37	..	Slight	34	..	Albumin +	57¾

* In this case, 0.1 c.c. injections were used previous to April 6.

edema, and also to the fact that in the same patients the disappearance time is considerably less than in the controls, even in the parts in which pitting is not present. From the later observation it would seem that this test may be of value in determining the so-called pre-edematous state of the tissues.

We plan to make a fuller report at a future date. We have also under consideration the testing out of solutions of different concentration and of different constitution, and the application of the test to cases showing dehydration.

The results so far obtained suggest that this test may be of value: first, in the detection of disturbed water balance in the tissue, and, secondly, as a means of following the progress of a case in which the presence of such a disturbance has been established.

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EXPERIMENTAL STUDIES ON INFLAMMATION.

II. EXPERIMENTAL CHEMICAL INFLAMMATION IN VIVO.

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In a previous paper the influence of various chemicals upon chemotaxis, *i.e.* migration of cells *in vitro*, was discussed.[1] In the present paper the capacity of these chemicals to produce the other features of inflammation, *i.e.* vascular changes, migration of cells *in vivo*, fluid exudation, and tissue repair, will be considered.

A review of the literature on the subject of experimental inflammation reveals little that throws light on the main problem; namely, what physical, chemical, and physicochemical properties determine that a given substance will or will not produce an inflammatory reaction. No systematic classification has been made of the different substances in regard to their ability to arouse inflammation. Most of the articles have been descriptions of the different cell forms and types of exudates occurring in inflammatory reactions. No definite distinction has usually been made between aseptic and microbic inflammation.

Sollmann[2] alone has attempted to compare chemical structure with a related process, urticaria, but was unable to establish any relation between chemical constitution and urticarigenic action.

Ikeda[3] tested many different substances injected by Cohnheim's method into the frog. He found that normally a certain number of cells migrates into the exposed mesentery from the intact blood vessels within ½ to 1½ hours, and those substances which decreased the number of cells were therefore considered as inhibiting to inflammation, those increasing the number as arousing inflammation.

[1] Wolf, E. P., *J. Exp. Med.*, 1921, xxxiv, 375.

[2] Sollmann, T., *J. Pharmacol. and Exp. Therap.*, 1916–17, ix, 391.

[3] Ikeda, Y., *J. Pharmacol. and Exp. Therap.*, 1916, viii, 101, 137.

The following substances were found to inhibit the cell migration produced by exposure to air: quinine, atophan, mercury perchloride, arsenic, antipyrine, morphine, chloral hydrate, and calcium chloride. There was a slight inhibition when urethane and ethylhydrocupreine were injected. There was no inhibition produced by cinchonine, scopolamine, atropine, strophanthin, colchicum, sodium salicylate, and magnesium sulfate.

The rest of the abundant literature on experimental inflammation, including the extensive studies of Cohnheim, Marchand, Maximow, and others, reveals nothing of significance in relation to our particular problems.

In the present work experimental inflammation in both frogs and mice has been studied.

Method.

Method Employed with Frogs.—Medium sized winter frogs were used, being kept in a cage so designed that there was a constant stream of fresh cold water through part of the cage, and Cohnheim's method with the frog mesentery was employed. The different substances examined were injected into the dorsal lymph sac before pithing the frog, or were dropped on the exposed mesentery after pithing. Both methods were tried with each substance to serve as a possible check.

As a preliminary test of the behavior of substances introduced into the dorsal lymph sac, colored oils, scarlet R in olive oil and Sudan III, were injected into this cavity. The colored drops could be seen on the exposed mesentery 5 to 10 seconds after injection. Methylene blue solution is visible 3 to 5 seconds after injection. Therefore dorsal lymph sac injection is practically the same as intraperitoneal injection, the fluid passing directly from the sac to the peritoneal cavity by way of lymphatic spaces. In the routine work the first examination was made 30 minutes after injection. If there was evidence of inflammation at this time, examinations were made on other frogs at the end of 20, 10, 5, and even 2 minutes after dorsal injection. If the reaction was very slight or absent at the end of 30 minutes, another frog was examined 1 hour after injection. If there was no evidence of inflammation at this time, the substance injected was arbitrarily classified as one producing no inflammation. Normally from three to five cells per low power field will migrate from the blood vessels into the exposed mesentery in ¾ to 1 hour. In warm weather there may be ten or twelve, and when the surrounding atmosphere is very dry and no attempt is made to moisten the mesentery, this may increase to twenty cells per field. The strength of the solutions varied from 0.005 to 20 per cent molecular concentration of the various substances when the formula was known, otherwise the concentrations were percentage by weight.

Method Employed with Mice.—White mice of medium size, weighing about 25 gm., were injected intraperitoneally under aseptic precautions. Each mouse was kept in a separate cage after injection and the different animals were ex-

amined at the end of 1, 12, 24, and 48 hours respectively, depending upon the reaction to the injected substance. At the end of these periods the animal was anesthetized with ether, and kept under surgical anesthesia throughout the examination. After surgical preparation a midline incision was made into the peritoneal cavity; this was opened carefully to avoid injuring the intestines. The intestines were minutely examined for gross lesions, the mesentery for abrasions, areas of dullness, or changes in color or other abnormalities. The different regions were then examined microscopically in the same way as the frog mesentery. It was impossible to make quantitative determinations of the increase in the number of cells in the mesentery and around the blood vessels because of the numerous cells present normally. Qualitative and comparative differences could be easily determined. During examination the mice could be kept under ether anesthesia for several hours if necessary, and the condition of the heart and the respiration carefully watched. The mesentery was kept moist with sterile physiological salt solution warmed to 37°C., when necessary. In all cases 0.25 cc. of a 1 per cent solution (molecular concentration when the formula was known, otherwise concentration by weight) was injected. When other concentrations were used, they are noted.

EXPERIMENTAL.

Sodium Salts.—The Na ion produced no inflammatory reaction *per se*, being tested as the chloride, tartrate, lactate, phosphate (mono-, di-, and tribasic), citrate, bicarbonate, and sulfate. Sodium cinnamate produced a very slight inflammatory reaction in both frogs and mice, if used in strong (15 per cent molecular) solution. Tribasic sodium phosphate caused some inflammation by virtue of its alkalinity. In frogs, sodium iodide caused a fluid transudate when concentrations over 5 per cent were used, but there were only a few cells outside the blood vessels.

Potassium Salts.—These had little more effect than the corresponding sodium salts, and no inflammatory action can be attributed to the K ion. The iodide caused more hyperemia than the other potassium salts, and in mice a slight leucocytic migration was observed, but not in frogs. The other salts, when concentrated (15 per cent molecular), caused some fluid effusion and a slight hyperemia.

Calcium Salts.—No calcium salt caused a marked inflammatory reaction. In frogs, calcium chloride caused a slightly increased blood flow in 25 minutes in concentrations of 10 to 25 per cent, and calcium citrate produced a slight migration of cells after 20 minutes, if concentrations of 10 per cent or over were used. When a 3 per

cent solution was injected into mice there was marked slowing of the blood stream, and in concentrations of 6 to 20 per cent the mice died of convulsions in 10 to 20 minutes. There were no gross lesions of inflammation at this time, and microscopic examination showed a normal mesentery.

Calcium carbonate, tartrate, and cinnamate caused only foreign body reactions. There was some hyperemia in mice injected with calcium lactate, while citrate caused some slowing of the blood and diapedesis, but no leucocytic migration. Calcium cyanide caused no inflammation when 0.25 cc. of a 0.3 per cent solution was injected; when larger amounts or higher concentrations were injected, the mice died within 10 to 15 minutes with no evidences of inflammation.

Magnesium Salts.—None of the magnesium salts caused any change in the frog mesentery, except the citrate; this caused an active hyperemia. In mice a slight hyperemia was observed with strong solutions.

Strontium Salts.—Strontium chloride caused no inflammation in the frog mesentery, but in mice there were some hyperemia and a slight cellular increase. When equal parts of strontium chloride and magnesium sulfate were used together, there was a slight inflammation as shown by hyperemia and three to seven cells per low power field in ½ hour in frogs. Similar slight effects were seen in mice, exceeding always the effects of either salt alone.

Barium Salts.—Barium sulfate and chloride caused no inflammation. When the solid particles were allowed to remain on the mesentery for several hours there was the typical reaction to a foreign body; *i.e.*, accumulation of cells around the foreign substance.

Mercury Salts.—Mercuric chloride caused no inflammation when a 0.5 or 1 per cent solution was injected into mice. When a 3 per cent solution was used there was a decided shrinking of the cells around the blood vessels and the mesentery was streaked with fibrin, but there was no true inflammation at the end of 24 hours. In higher concentrations the animals died within 24 to 36 hours with no evidences of mesenteric inflammation. Mercurous nitrate caused a very slight inflammatory reaction at the end of 1 hour; after 24 hours the mesentery looked red grossly and on microscopic examination there were many leucocytes, round cells, fibroblasts, and large oval or round cells with many nuclei. Mercuric subsalicylate produced no inflammation.

Free Organic Acids.—Lactic acid, nucleic acid, and uric acid caused no inflammation.

Oleic acid caused a decided dilatation of the intestinal and mesenteric vessels when a 5 per cent solution was injected in mice. The mesentery appeared cloudy, grossly. Microscopically there were fibrinous strands deposited around the blood vessels and an increase in the number of cells in the mesentery.

Amino-Acids.—In mice, glycine produced a slight inflammation at the end of 1 hour. With alanine the rate of flow was decreased but there were a few more cells free in the mesentery than normal. Phenylalanine, glutaminic and aminovalerianic acids, and tryptophane caused a slight increase in the number of polymorphonuclear leucocytes in the mesentery. Histidine caused no inflammation, but there was some diapedesis of erythrocytes. Tyrosine hydrochloride caused a dulling of the mesentery on injection; microscopically there were masses of white blood cells infiltrating fibrinous strands, but there was no increase in the amount of blood present. None of these amino-acids caused any inflammatory reaction in frogs.

Tyramine.—This compound was used in concentration of 1 per cent.[4] It was neutralized with 0.01 N NaOH, with phenolphthalein as an indicator (pH 7.7). In frogs it caused no true inflammation, but the white blood cells were clumped together in all the mesenteric vessels in small groups, at the end of about ½ hour, and there were red blood cells caught with the white blood cells, forming thrombi. The rate of flow of the blood was about normal for 25 minutes after injection, then the rate decreased, and became sluggish, and finally stopped. This clumping of the erythrocytes had previously been observed *in vitro*[1] and at that time no explanation could be given. It seems to affect the blood of different species in the same manner, in the *in vitro* work at least.

In mice, tyramine produced marked changes in the mesentery when 0.5 cc. of a 0.95 per cent solution of the hydrochloride was injected. Grossly the blood vessels appeared dilated at the end of 45 minutes. Microscopically there were groups of red blood cells clumped together within the blood vessels, particularly at the bifurcation of the smaller

[4] The tyramine was made synthetically and was provided by Dr. M. T. Hanke and Dr. K. K. Koessler.

veins, though there were some within the arterioles. In the small capillaries there was no circulation of the blood because of thrombi, although occasionally a thrombus would break loose and some few cells pass through, soon to be stopped by another clump of red blood cells. There were a few more extravascular cells than usual, but only the amount found in slight inflammation. At the end of 24 hours the capillaries showed only masses of clotted erythrocytes and there was no circulation in these vessels. In the mesentery there were some young connective tissue cells, some fibrin, and a few polymorphonuclear leucocytes. 48 hours after injection the flow of blood was still more sluggish than normal because of clots present at the bifurcation of the smaller vessels and in some of the capillaries. These were no longer the agglutination thrombi seen earlier but true clots with fibrin, red blood cells, and white blood cells. The mesentery was approximately normal, but there were more fibroblasts than normal and some early connective tissue cells. This is similar to the results obtained with frogs, although in these the predominating cell in the clumps of cells were white blood cells. With the mouse the clot was composed almost entirely of erythrocytes, white blood cells and fibrin being present only late, and in small amounts.

Histamine.—In concentrations of 0.1 to 0.25 per cent histamine caused marked inflammation when injected into the dorsal lymph sac of frogs. A set of observations is given.

9.00 a.m. Injection of 1 cc. of 0.25 per cent solution of histamine.

9.02 a.m. Increased rate of flow with an occasional white blood cell outside the contracted blood vessels.

9.05 a.m. Blood vessels are less contracted, from nine to twenty white blood cells outside the blood vessels per low power field.

9.10 a.m. More cells are outside the blood vessels, from twenty to twenty-five per low power field, the blood vessels are distended, and the rate of flow is increased.

9.15 a.m. White blood cells are scattered through the entire mesentery, some erythrocytes can be seen leaving the small capillaries by diapedesis, and the rate of flow is increased.

9.20 a.m. Frog died; heart stopped beating.

When histamine was applied directly to the frog mesentery the results were similar to those obtained on injection. Stained specimens made at different times showed deposition of fibrin strands

throughout the mesentery, and a thick layer of fibrin covered the mesentery at the end of 10 minutes, with many leucocytes and erythrocytes in the meshes. This action was in keeping with the marked positive chemotaxis that histamine showed in all the *in vitro*[4] work, when low concentrations were used.

In mice, histamine produced an inflammatory reaction at the end of 1 hour when 0.25 cc. of a 0.25 per cent solution was injected. It was found at the end of 15 minutes that the blood vessels were smaller than normal, and on microscopic examination the lumen was much reduced in size, much more so than the gross appearance of the vessel suggested. There were no evidences of inflammation at this time. 45 minutes after injection the mesentery appeared red, and the blood vessels were distended. Microscopically there were some few cells free in the mesentery, many of them being erythrocytes. These were most numerous around the smaller blood vessels, and some were in clumps around the capillaries. At the end of 1 hour, there were many more wandering cells free in the mesentery than before, and there was marked diapedesis of red blood cells. At the end of 24 hours, there was no acute inflammation but there were fibroblasts present throughout the mesentery, and a few polymorphonuclear leucocytes and some erythrocytes. At the end of 48 hours, there were still a few groups of polymorphonuclear leucocytes and an increase in the fibroblasts and young connective tissue cells. There were areas where erythrocytes had accumulated outside the blood vessels; the cell outlines were indistinct and broken. This is to be expected from the results obtained in frogs, although with frogs the reaction was more rapid and severe. *In vitro* work with histamine showed that the leucocytes of different animals react differently with histamine even under the same conditions, so that the present results are not surprising.

Since this work was done, the report of Bloom[5] has been published. He concludes that histamine does not produce inflammation when injected in cats, though there is a transudate which is most marked at the end of 1 hour. In mice the maximum action occurred at the end of an hour. When collodion capsules containing histamine, 1:100 in physiological saline, were placed between the internal and external oblique muscles in cats, there was a marked inflammatory

[5] Bloom, W., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 185.

reaction, but this was in no way different from that caused by collodion capsules containing normal saline, though very different from that caused by collodion capsules containing lactic acid, which is negatively chemotactic. When using open glass tubes containing histamine, *in vitro* and *in vivo*, Bloom found no attraction of leucocytes by the histamine; *i.e.*, no positive chemotaxis. His findings are contrary to those obtained by us;[1] and Sollmann[2] found histamine to be one of the most active agents (1:500,000) in producing urticaria, which is a process closely related to inflammation.

Alkaloids.—None of the following alkaloids caused inflammation: morphine and morphine sulfate, codeine and codeine hydrochloride, quinine sulfate and bisulfate, strychnine sulfate, brucine sulfate, caffeine sulfate and citrate, heroine and heroine sulfate.

All quinine salts caused a slowing of the blood stream and fewer cells migrated into the mesentery from the blood vessels 1 hour after exposure to the air, but the difference was so slight that it is impossible to say that quinine inhibits inflammation.

Ethyl Alcohol.—No inflammation was caused in mice in concentrations below 10 per cent, but in concentrations of 5 to 25 per cent there was a slight migration of white blood cells. In concentrations of 25 to 30 per cent there were many white blood cells outside the blood vessels, from seven to twenty per low power field. These were smaller than normal, and the entire mesentery appeared dry. When fixed and stained, there were strands of fibrin present and the mesentery appeared dehydrated.

Ethyl Acetate.—This compound acted like ethyl alcohol, the inflammation being negligible. It was used as a solvent for cantharidinum.

Ether.—Ether acted like ethyl alcohol, but a 1 per cent concentration acted like the 10 per cent solution of alcohol. There was marked dehydration of the frog omentum in higher concentrations.

Urotropine.—Urotropine, or hexamethylenamine, caused a mild inflammation in frogs with concentrations of 5 per cent at the end of ½ hour if the media was acid. When the mesentery was neutral or alkaline there was no reaction.

Parazol.—This substance (crude dinitrodichlorobenzene)[6] in concentrations of 1 per cent or less caused a slowing of the blood stream of the frog omentum and then in many instances the heart stopped

[6] Wells, H. G., *J. Indust. Hyg.*, 1920, ii, 247.

beating. There were a few white blood cells outside the blood vessels. Stained specimens showed a marked deposition of fibrin through the mesentery, and the cells were indistinct, with poorly stained nuclei. In mice severe typical inflammation was produced by 0.2 cc. of a 0.3 per cent aqueous or oily solution, accompanied by much necrosis. This is somewhat similar to the action of parazol *in vitro*, in which high concentrations cause a negative reaction (chemotaxis) with a necrotizing action, while low concentrations are positively chemotactic.

Papain.—Papain, or papayotin, acted differently in winter and in summer frogs. In the former there was no inflammation when a 20 per cent solution of papain was injected into the dorsal lymph sac or when it was applied directly to the exposed mesentery. When summer frogs were used or when winter frogs were kept in a warm place for several hours, so that the body temperature was over 85°F., there was a definite inflammation on the injection of a 20 per cent solution of papain. This is similar to the results obtained *in vitro*, when the blood of summer or winter frogs was used, the former being positively chemotactic, the latter negatively chemotactic.

In mice, papain, or papayotin, caused a slight inflammation at the end of an hour. There were many white blood cells throughout the mesentery. At the end of 24 hours, the intestines were red, and the mesenteric vessels were distended. Microscopically there were many more wandering cells around the blood vessels than normal and ameboid movements could be seen. In the mesentery there were groups of cells, largely polymorphonuclear leucocytes, though some fibroblasts were present, and in the meshes of fibrin a few erythrocytes. At the end of 48 hours, there were many connective tissue cells with interfibrillar substance, and a few large cells with faintly staining nuclei, epithelioid cells, around the red blood cells. This was similar to the results obtained with summer frogs and is to be expected from the *in vitro* work with the blood of warm-blooded animals.

Cantharidinum.—Cantharidinum (U. S. P.), obtained from Chinese cantharides, caused marked inflammation in the frog mesentery. This drug was dissolved in 5 to 10 per cent ethyl acetate in water. The following is a typical protocol.

8.00 a.m. Injected 1 cc. of a 2 per cent solution of cantharidin into the dorsal lymph sac.

8.02 a.m. Rapid rate of flow of blood.
8.05 a.m. From two to five cells outside the blood vessels per low power field.
8.10 a.m. Marked distention of blood vessels; from nine to fifteen white blood cells per low power field.
8.12 a.m. Increasing number of white blood cells emerging from blood vessels.
8.20 a.m. Many more cells outside the blood vessels, from 70 to 100 per low power field; diapedesis of erythrocytes.
8.30 a.m. Too many cells outside the blood vessels to count; blood vessels contracting slightly.
8.40 a.m. Heart stopped beating.

All the mesenteries were stained and it was found that at the end of 5 minutes after injection the walls of the blood vessels were thinned and strands of fibrin could be followed along the blood vessels. At the end of 15 minutes, the fibrin was present in sheets and many polymorphonuclear leucocytes, red blood cells, and a few lymphocytes were free in the mesentery.

In mice, cantharidinum produced marked inflammation when 0.25 cc. of a 0.5 per cent solution was injected. The mice were unable to stand. Gross examination revealed a yellow exudate at the end of ½ hour. Microscopically there were many polymorphonuclear leucocytes in the mesentery, and fibrinous strands along the blood vessels with erythrocytes in the meshes. At the end of 1 hour, the entire mesentery was covered with polymorphonuclear leucocytes and erythrocytes, and in some places the fibrin was so thick that the cells were hard to see clearly. At the end of 24 hours, there was no exudate present, but microscopically there were groups of polymorphonuclear leucocytes around clumps of fibrin in certain areas. There were many fibroblasts. At the end of 48 hours, the fibroblasts had increased markedly and the polymorphonuclear leucocytes had decreased, the fibrin was diminished, and there were only a few erythrocytes present. 72 hours after injection, there was little evidence of inflammation; the increase in fibroblasts and immature connective tissue alone showed that there had been an inflammation.

Croton Oil.—This substance caused severe inflammation. Some consider that the active principle of croton oil is an acid,[7] others

[7] Bastedo, W. A., Materia medica; pharmacology; therapeutics; prescription writing, for students and practitioners, Philadelphia and London, 2nd edition, 1918, 136.

that it is a resinous anhydride. When 0.25 cc. of a 1 per cent solution in olive oil was injected into mice there was no inflammation at the end of 1 hour, but within 6 hours there were many white and red blood cells in the mesentery and a marked increase in the rate of flow of the blood. At the end of 12 hours, the entire mesentery and intestines were red, and in places the peritoneum was no longer smooth and shining, though there were no definite areas of exudation. There were many cells in the mesentery. At the end of 24 hours, the individual oil drops were surrounded by numerous cells and in many instances by fibrinous strands. There were a few fibroblasts at the outer edges of these clumps of cells. At the end of 48 hours, there were many fibroblasts, many of the polymorphonuclear leucocytes had disappeared, and the oil drops were fewer in number and smaller in size. Young connective tissue cells were present, though not numerous. At the end of 72 hours, there were many fibroblasts and connective tissue cells, but few other evidences of inflammation. These cells were abundant around the traces of oil that were still left. When higher concentrations were used, there was more active, early inflammation, with more polymorphonuclear leucocytes, and many erythrocytes were present in meshes of fibrin.

In frogs similar inflammatory reactions were produced. With solutions over 10 per cent the frogs had convulsions in spite of pithing and at the end of 20 minutes the blood vessels to the mesentery were so torn that it was impossible to examine the tissues under the microscope. Stained specimens at the end of 5 minutes showed large amounts of fibrin in the mesentery and some few polymorphonuclear leucocytes.

Turpentine.—Marked inflammation was induced with concentrations of 0.5 to 5 per cent in olive oil. When higher concentrations were used in frogs the blood vessels ruptured before the animal could be examined. Stained specimens showed strands of fibrin along the blood vessels at the end of 10 minutes, and almost a complete sheet of fibrin over the mesentery at the end of 25 minutes. In mice typical inflammatory reactions were produced.

Jequirity.—A preparation made by grinding the jequirity seeds, adding water, and shaking in a shaking machine for 24 hours caused only a slight inflammatory reaction; indeed there was just about

the number of cells free in the mesentery that is usually found after exposure to air. This was repeated many times with seeds from three different sources, and the same results were obtained, despite the reputation this seed has for producing profound inflammatory changes.

Miscellaneous Substances.—Urethane, urea, creatinine, olive oil, veronal (diethylmalonylurea), yeast vitamine (Harris), olive oil, chloretone, glucose, mercurochrome 220, Witte's peptone, egg albumose, and Armour's peptone caused no inflammation in either frogs or mice. In mice, acetanilide caused a slight inflammation at the end of 8 hours.

The effect of aspirin (acetyl-salicylic acid) was peculiar when the usual amount, *i.e.* 0.25 cc. of a 1 per cent solution, was injected. The animals died within 10 minutes, with no lesions to account for death, and no inflammation was present. When 0.1 cc. was injected, there was no evidence of inflammation at the end of 24 hours.

Scarlet R, purified, caused no inflammation at the end of 1 hour, but 24 hours after injection, there were many erythrocytes and polymorphonuclear leucocytes around the individual oil drops. At the end of 48 hours, there were many fibroblasts, but no true inflammation.

DISCUSSION.

Cohnheim's method for the study of aseptic inflammation gives very satisfactory results for *in vivo* work. Definite quantities can be injected and a microscopic examination made of the mesenteries.

The results may be determined in a definitely quantitative manner by counting the cells migrating out of the blood vessels in a given field, and measuring the increase in size of the blood vessels by the fine screw on the microscope, when frogs are used. With mice, qualitative results only can be obtained; quantitative results are impossible, because of the normal cellularity of the mesentery.

The different types of cells can be detected easily without fixing or staining, but fixing with Zenker's fluid and staining with hematoxylin and eosin make the different cells more distinct; also the changes in the walls of the blood vessels and the amount of fibrin are striking. The migration of the leucocytes through the capillary walls can only be seen distinctly during the life of the animal, before fixation and the subsequent shrinkage.

Dorsal lymph sac injection in frogs is practically the same as intraperitoneal injection; this was clearly seen when colored fluids were injected, as a watery solution of methylene blue, or an oily solution of scarlet R, which make their appearance in the peritoneal cavity almost immediately.

The body temperature of the animal seems to be of importance with certain substances, particularly papain. In the majority of instances, however, it makes no difference. Substances which react differently at different temperatures *in vivo* are found to be those which reacted differently towards the blood of cold- and warm-blooded animals *in vitro*.

CONCLUSIONS.

1. None of the salts tested produce a marked inflammation *in vivo* in concentrations under 10 per cent. Potassium salts and the different citrates produced atypical inflammatory reactions in mice, but not in frogs. There was no true inflammation, however, characterized by blood vessel changes, migration of polymorphonuclear leucocytes and erythrocytes, and fluid exudation.

2. Synergistic action occurs when equal parts of strontium and magnesium salts are employed. There is a change in the appearance of the mesentery without a true inflammation, and this change does not occur with either salt alone

3. Amino-acids and amines as a class do not produce inflammation, but histamine produces a marked inflammatory reaction in frogs and mice.

4. Tyramine does not cause an inflammatory reaction but has other marked effects; agglutination thrombi occur within the smaller blood vessels, both veins and arteries; in frogs there is a rapid clumping of the white blood cells followed by a true coagulation with strands of fibrin and entanglement of erythrocytes. This is very widespread and often kills the animal within an hour after injection. In mice it is the erythrocytes that clump and coagulation occurs very much later, usually at the end of 24 hours; still later there is complete absorption of the coagulated masses and the mesenteric circulation returns to normal. None of the mice died during the stage of clumping, and the clots never extended up the larger vessels as they did in the frogs.

These effects are similar to the phenomena observed in the *in vitro* work, in which clumping of the cells appeared constantly.

5. Cantharidinum, histamine, and turpentine produced the most rapid and marked inflammation of any substances tried. These substances are all strongly positively chemotactic *in vitro*. The differences occurring when these substances are used in different species is a quantitative rather than a qualitative one, the body temperature being of some importance. Papain acted only in warm-blooded animals; this is consistent with its chemotactic action *in vitro*. The degree of positive chemotaxis varied markedly with the blood employed and in the *in vivo* work the inflammation varied with the species of animal used.

6. Certain substances produced inflammation only some time after injection; this is true of scarlet R and croton oil in weak dilutions. These are not strongly positively chemotactic.

7. Parazol produces an inflammation associated with necrosis of the tissues. This is similar to the results obtained *in vitro*, parazol being positively chemotactic in low concentrations and negative in high concentrations.

8. The exact chemical nature of many of the substances which produce marked inflammation is unknown. This is true of cantharidin, and the active constituents of turpentine and croton oil.

9. All substances which produce marked and rapid inflammation on injection are positively chemotactic, but not all strongly positively chemotactic substances produce inflammation; *i.e.*, calcium compounds, sodium phosphate, etc.

10. Only substances which are positively chemotactic and also soluble in oil seem capable of producing inflammation in animals.

BLOOD TRANSFUSION BY THE CITRATE METHOD IN HEMORRHAGES OF THE NEW BORN*

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The use of whole blood injections is a well recognized clinical procedure in cases of hemorrhage occurring in new-born babies. Several methods have been devised for this procedure, all of which have certain features to recommend them. The simplest method is the use of subcutaneous or intramuscular injections, from 10 to 20 c.c. being employed at a time. These are often repeated two or three times in a given case, if the primary injection is not successful in arresting the hemorrhage. This is a perfectly safe method, and can be carried out under any circumstances by a physician of ordinary skill. The objection to its routine use is that frequently cases fail to respond favorably; moreover, such injections are frequently painful and irritating to the recipient.

A second method is the direct transfusion through anastomosis of veins by means of cannulas, the median basilic vein of the donor being united to the external jugular of the baby. This method is good, but is rather cumbersome. It requires the use of trained assistants, and not a little skill in blood vessel surgery for its successful accomplishment. The amount of blood transferred is not accurately measured, but must be judged by the change in color of the baby and thus approximated. It has the advantage that nothing but blood is injected, which avoids all possible danger of toxic reaction due to anticoagulants.

A third method is the injection into the longitudinal sinus by means of syringe or buret of whole blood, either with or without the addition of citrate to prevent clotting.

This method is used especially by pediatricians, and in the hands of one doing this kind of work is doubtless very satisfactory. For the general practitioner, however, the procedure is considered too formi-

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dable unless he has had some special training along these lines. The objection advanced is that one cannot be sure that the needle is in the lumen of the sinus throughout the injection, even if it is there when the injection is begun. I have seen one case at necropsy, after injection by a man who claimed that he had had considerable experience in the use of this method. A large hematoma was found under the dura. The needle had evidently penetrated the side wall of the sinus after the injection was begun. Numerous other reports of similar accidents are on record.

Fourthly, various substances other than whole blood have been used in this type of case, with indifferent results: horse serum, blood coagulants and human serum, either of the father or the mother of the child or of some person not a blood relative. The general experience has been that they work very well in the mild cases, but frequently fail in the severe form of the disease.

It would seem, therefore, that the use of whole blood in these cases is the only reliable way to stop the bleeding. If this could be done in a simple and safe method that is not too complicated for the average surgeon, a marked advance would be made in the treatment of these cases. It was in the hope of developing such a method that I undertook this piece of investigation.

It seemed reasonable that, if the peripheral veins were not too small, their use would be advisable, because any one familiar with the procedure in adults would be better able to acquire the technic in babies. In discussing the question with the pediatricians, however, I was told that the peripheral veins were too small, and that the method was impracticable. The same opinion was advanced by the men doing the direct transfusion. However, the dissection of a number of veins in bodies that I saw postmortem proved beyond a doubt that these veins could be used if the proper instruments and technic were employed. Losee [1] mentions this fact in an article on the use of blood transfusion in cases of severe hemorrhages in infants. He does not describe his technic. Bamberger [2] describes a case in which he successfully used the median basilic vein.

The median basilic vein was first selected as most accessible and presumably the most satisfactory. After it had been used on several occasions, however, it

1. Losee, J. R.: Bull. Lying-In Hosp., New York **12**: 100 (July) 1922.
2. Bamberger, A.: Illinois M. J. **39**: 27 (Jan.) 1921.

proved to be so small that a very fine needle had to be used to insert into the vein. This resulted in a very slow flow of blood, when the gravity method was used, making the procedure very tedious and sometimes resulting in coagulation of the blood before the completion of the operation. These difficulties were especially seen in premature infants.

INSTRUMENTS

Two pairs of fine rat-tooth tissue forceps	One luer
Two pairs of mosquito artery forceps	One rubber catheter, 18 inch, No. 25 connection
One pair of iridectomy scissors	One constrictor for donor's arm
One scalpel	One small syringe for injecting procain
One large bore bleeding needle	One skin needle and hair
One pair of ordinary scissors	Ten c.c. of a 2 per cent solution sodium citrate
One Luer needle No. 19	One tube of No. 0 catgut
One 4-ounce glass graduate and stirring rod	Special board to hold baby

The external jugular vein was therefore selected because of its size, and also because of its accessibility. It is located just under the platysma muscle, and can be easily exposed by a small incision through the skin and muscle. It can readily be seen when the baby cries. The femoral or long saphenous vein has been mentioned in this connection, but was not used in this series because of the inevitable soiling of the wound postoperatively by urine and feces.

The citrate method was selected because of its simplicity, and because probably more men are familiar with its use than with other methods. There is very little, if any, danger of a toxic reaction from the small amount of the drug used in a single injection.

TECHNIC

Since the object of this work was to develop a method that would be practicable under almost any circumstances for men of average skill, the equipment has been made as simple as possible for efficient work. Only a few instruments are necessary, but they must be good and of the right kind.

For the special holding board, an inch board about 20 by 10 inches is used as a base. Across this, about 4 inches from one end, is set another piece of inch board 2 inches high, called the neck board. A series of ½ inch holes are drilled in the base board about 2 inches from the edges and about 4 inches apart, through which bandages tied to the baby's ankles and wrists can be passed. The baby is thus secured to the board.

The baby is placed back down on the board so that the neck board comes under the back of its neck, and the hands and ankles are secured as mentioned above.

The head is then held rotated to one side by a nurse or assistant. This exposes the region of the opposite external jugular vein. In cases in which transfusion is indicated, the condition of the baby is such that it offers, in most cases, little resistance or objection to being placed on the board.

It is advisable to have a long narrow bore glass tube as a buret, and we find that the glass portion of the Asepto irrigating syringe works very well. We have also used the barrel of an ordinary 20 c.c. Luer syringe for this purpose. The object of having the buret long and slender is to facilitate observation of the rate of injection, since the flow of blood through the fine needle is necessarily slow.

The needle used must not be sharp pointed because of the tendency to cut and tear the wall of the vein. For this reason the ordinary Luer needle is made blunt by grinding the point down with an emery wheel.

Before the operation begins, it is well to have everything in shape to complete it without delay. The importance of this point is that if there are delays, a certain amount of clotting is liable to occur, which interferes with the flow of blood through the small needles that it is necessary to use in this work.

The donor's arm is prepared for venipuncture in the usual way, and is painted with iodin. A 12 inch No. 0 catgut ligature is doubled, and the ends are grasped by mosquito forceps. The skin over the external jugular is painted with iodin followed by alcohol, and the region of the vein injected with a small amount of procain or apothesin 1 per cent. solution. The vein can be readily seen in most cases, especially when the baby cries.

A few minutes are allowed for the anesthetic to take effect, and then a small incision is made about an inch in length along the course of the vein through the skin and platysma muscle. In infants that have had a severe hemorrhage, the vein may be hard to find, owing to the collapsed condition. The baby will usually cry, however, and this will give the needed distention. After exposure, the vein is grasped very gently with fine tissue forceps, and carefully cleaned of perivascular connective tissue. The importance of this point is that the vein cannot be properly opened, nor the needle easily inserted, unless the vein wall is quite free. The vein is next lifted up, anl the double ligature passed underneath. This is sometimes facilitated by passing a grooved director before passing the ligature. The loop in the catgut is then cut, giving two ligatures under the vein. The ends of each ligature are caught in mosquito forceps. One ligature is drawn upward and the other downward so that the segment of the vein that is to be opened is raised clear of the wound, and all circulation in it is stopped. At this point the vein is dropped back into the wound, and a pad soaked with salt solution is placed over the incision to prevent drying

The blood is now obtained from the donor in the usual way, a large bleeding needle being used with a 6 inch rubber tubing attached, into the 4 ounce graduate containing 10 c.c. of a 2 per cent. solution of sodium citrate. The blood is constantly stirred during collection, and until it is ready to be poured into the buret, thus insuring thorough mixture with the citrate solution. In donors with fine veins or in subjects in whom the arm is very fat, conditions frequently encountered in women, the vein is transfixed by a fine cambric needle to prevent rolling when the puncture is made.

After the collection of the blood, the salt solution pad is removed from the baby's neck, and the segment of the vein between the ligatures is raised clear of the wound. The vein is next incised half way through its diameter with the fine iridectomy scissors. Ten cubic centimeters of physiologic sodium chlorid solution is placed in the buret, which is connected to the No. 19 Luer needle by an 18 inch rubber tubing about the size of the ordinary rubber catheter. This needle must have a blunt point, which prevents injury to the vein wall during the insertion. When it is seen that the salt solution is flowing freely through the needle, the opened segment of the vein is lifted up, and a stream of salt solution from the needle is directed at the hole in the vein. This dilates the hole and materially aids in the introduction of the needle into the vein. The proximal ligature is slightly relaxed as the needle enters the vein, and then drawn taut after the insertion of the needle. The needle is thus held in place.

The salt solution is allowed to flow into the vein; when it is seen that a good flow is established, the citrated blood is poured into the buret by the assistant, and the buret kept filled until all of the blood has been injected. The last of the blood is followed by a few cubic centimeters of salt solution. The needle is then withdrawn, and the vein is tied off above and below the opening with the tension ligatures mentioned above.

The wound is closed by one catgut ligature in the platysma and a subcuticular horsehair suture in the skin. It is dressed with a dry dressing, which may be changed to a hot boric compress if infection occurs, as happened in one of these cases. If everything works smoothly, the transfer can be effected in about fifteen minutes from the time the collection of the blood begins.

The donor in these cases may be the mother, the father, or even some persons not a blood relative of the baby.[3] There is no necessity to group the blood of the donor or recipient, as it has been shown that the hemagglutinins and precipitins are not developed in the child to any great extent until it is about 2 years old. It was found, in this series, that sometimes the blood of one donor was more effective in controlling

[3] Hopp: J. Exper. Med. 31: [illegible]

the hemorrhage than that of another. Blood from the mother frequently seemed to be better than that from the father.

There does not seem to be any danger of overloading the heart in giving the blood in this way, as the flow into the vein is very slow, and in none of the babies was there any evidence of respiratory or circulatory embarrassment. Many of the babies had an elevation of temperature of a few degrees for a few hours, but seemed to be otherwise none the worse for the operation.

RESULTS

In all, there were fourteen cases in this series. The seriousness of the hemorrhage varied from a slight capillary oozing from a mucous membrane to a severe anemia which resulted from extensive bleeding from the cord, or from a melena. In many of the cases, various remedies had been tried before transfusion was undertaken, such as retying and sewing the umbilicus in cord hemorrhages, or the giving of various other forms of coagulant, such as blood coagulants, horse serum, or human serum under the skin. These measures had failed to produce the desired effect, and so transfusion was resorted to. The operation was followed by recovery in all cases, but in a few it was necessary to repeat the transfusion.

In one case a baby was suffering from a severe icterus neonatorum, and the jaundice persisted for several days after the hemorrhage was controlled. This is suggestive of the possible value of this procedure in severe cases of this kind unaccompanied by actual hemorrhage.

Clinically, striking improvement is shown by these babies after they had received from 75 to 100 c.c. of blood. The color is better, the bleeding usually stops in a few minutes, and the child is very much more active. It can be put to the breast in a short time, and is usually vigorous enough to take its feeding. If it is very weak from blood loss, it is probably better to feed it breast milk from a bottle for some time until it regains some of its strength, and is free from the danger of recurrence of the hemorrhage. If recurrence does occur, it may be necessary to repeat the process. In this case, if one external jugular has been used, it is probably better to use the median basilic vein, if it is not too small.

This, however, is not always necessary, as in one case I transfused a baby that had had a previous trans-

fusion of defibrinated blood into the external jugular. This child started to bleed again on the second day, and I attempted to use the opposite external jugular for a second transfusion. As a result of defective apparatus, the blood clotted while we were getting it into the vein, and we were forced to tie off that vein. We then tried the arm vein, but it was too small, and broke while we were inserting the needle. I therefore reopened the wound I had made in the neck and dissected out the internal jugular vein, passed ligatures in the usual way, injected the blood, and tied off that vein. This left the baby with both external jugular veins and one internal jugular vein tied off; but, as far as could be seen, it did not affect the circulation of the head in the least, and the baby made an uneventful recovery.

One point that might be mentioned that we have learned from these experiments is that in using blood from the mother it is better to allow a slight excess of citrate solution, as it would appear that more is necessary to prevent coagulation in mother's blood than in blood taken from the father.[4] This clinical fact is in line with some results that I have obtained in the study of the coagulation time of the blood in puerperal women.

These hemorrhages occurred from the first to the fourteenth day of life, and are more common about the fourth to the seventh day. In the melena cases there may be no evidence of bleeding for some time, except the pallor and weakness of the baby, until a large, black stool with or without fresh blood reveals the diagnosis.

Bleeding from the cord or from a circumcision is usually detected early, but much valuable time is frequently lost trying ineffectual methods to control it, such as retying the cord, putting in extra stitches, or the use of styptic applications locally. These hemorrhages are chemical in origin, and cannot be controlled by mechanical means. The only sure way of controlling them is by the administration of whole blood, and the best way of accomplishing this is by the intravenous blood transfusion of citrated blood.

It is not known just what happens in the blood of new-born babies that results in the failure of the normal mechanism of coagulation to function. Rodda[5] has shown that there is a decrease in the coagulability,

4. Falls, F. H.: J. A. M. A. **79**: 1816, 1922.
5. Rodda, F. C.: Determining Coagulation Time of Blood in New Born, Am. J. Dis. Child. **19**: 268 (April) 1920.

beginning with the second day and reaching its lowest point about the fourth or fifth day. The coagulability returns to normal in most cases about the eighth to the tenth day, and the bleeding time as well as the coagulation time may be prolonged in babies apparently otherwise normal.

It would seem possible or even probable that this phenomenon is intimately related, either as a result of, or as part of, the process of icterus neonatorum. This process occurs, and disappears in about 50 per cent. of cases at the same time as the decrease in the coagulability is manifest, and its cause has never been satisfactorily explained.

It is obvious that something is lacking in the blood of these infants which is essential to normal blood coagulation and which predisposes to these hemorrhages. Whatever this substance or these substances are, they are present in excess in normal adult blood and are not dependent on the calcium content of the donor's blood, for in these experiments the injected blood contained its calcium supposedly in the form of the insoluble calcium citrate. It is more likely, therefore, that some of the other constituents of the blood entering into the coagulation are deficient, such as the thrombokinase or prothrombin, or that some substance in the blood, such as bile, interferes with the normal activity of the coagulation ferments.

CONCLUSIONS

1. Blood transfusion by the citrate method is a specific for the treatment of the hemorrhages of the new-born.

2. Peripheral veins, and especially the external jugular veins, are well adapted for this procedure if the proper instruments and technic are applied.

3. This method is safer than the injection of blood into the longitudinal sinus, and is not too technically difficult for the average physician.

4. The recovery of a patient with severe icterus neonatorum suggests the use of this procedure in this condition.

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